AWARD NUMBER: W81XWH-14-1-0492

TITLE: Differences between Human and Rodent Plasmacytoid Dendritic Cells May Explain the Pathogenic Disparity of Hantavirus Infection

PRINCIPAL INVESTIGATOR: Vincent Lombardi

CONTRACTING ORGANIZATION: WHITTEMORE PETERSON INSTITUTE FOR NEURO-IMMUNE DISEASE
RENO NV 89557-0001

REPORT DATE: June 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2016	Final	30Sep2014 - 29Mar2016

4. TITLE AND SUBTITLE
Differences between Human and Rodent Plasmacytoid Dendritic Cells May Explain the Pathogenic Disparity of Hantavirus Infection

5a. CONTRACT NUMBER
#W81XWH-14-1-0492

5b. GRANT NUMBER
PR131133

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Vincent C Lombardi

E-Mail: vclombardi@nvcbr.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
WHITTEMORE PETERSON INSTITUTE FOR NEURO-IMMUNE DISEASE
RENO NV 89557-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Unlike asymptomatic infection in rodents, hantaviruses cause two acute febrile diseases in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardio-pulmonary syndrome (HCPS). The purpose of this project is to evaluate the ability of hantaviruses to infect human and murine plasmacytoid dendritic cells (the primary circulating producers of type I interferon) and to evaluate the role of pDC granzyme in the pathogenesis of hantavirus infection. Our overreaching hypothesis is that the disparity between the asymptomatic rodent infection and the pathological human infection is a result of differences between how these cells respond to infection. The scope of this work will primarily encompass transcriptional analysis of pDCs isolated from human and mice using RNAseq and confirmed by quantative PCR.

15. SUBJECT TERMS
Hantavirus, granzyme B, RNAseq, plasmacytoid dendritic cell

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	38	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

The purpose of this project is to evaluate the role of plasmacytoid dendritic cells (pDCs) in the pathogenesis of hantavirus infection in human and small rodents with an emphasis on pDC-derived granzyme B (GZMB) expression. It is well documented that hantavirus infection causes acute, often fatal, infection in human [1]; however, infection of small rodents is usually asymptomatic and does not affect the animal's life span [2, 3]. Our overreaching hypothesis is that differences in clinical presentation could be based on the ability of hantaviruses to activate GZMB in pDC. Furthermore, we propose that hantaviruses have the capacity to infect human pDC and activate GZMB, thus initiating an inflammatory immune response that leads to the severe clinical presentation associated with hantavirus infection in humans. We further hypothesize that pDCs from small rodents do not activate GZMB to the same extent. If confirmed, this may provide an explanation, at least in part, for the different clinical presentation in humans and small rodent. Additionally, if confirmed, these data would identify GZMB as potential target for developing future diagnostics and therapeutics.

2. KEY WORDS:

Hantavirus; granzyme B; GZMB; RNAseq; HFRS; HPS; plasmacytoid dendritic cell

3. OVERALL PROJECT SUMMARY

Transcriptome analysis

In order to explore the contribution of pDCs to hantavirus pathophysiology, it was our goal to conduct transcriptome analysis, by next-generation RNA sequencing (RNAseq), of purified human and murine pDCs upon infection with pathogenic and non-pathogenic strains of hantaviruses. This method would allow us to potentially and unbiasedly identify differentially expressed genes that were a result of differences between humans and rodents as well as differences between viral strains. pDCs typically represent less than 0.3% of circulating lymphocytes [4], therefore, for each experiment, a unit (aprox. 450 mL) of whole human blood is required or the spleens from at least four mice. Each purification experiment of human blood yielded approximately 1 million cells, which were split between three virus strains and a control, giving approximately 250,000 cells per experiment. The yield from the murine spleens was approximately 200,000 cells per purification giving approximately 50,000 cells per experimental condition. Because of the limited amount of biological material, and other possible factors not observed when other cell populations were used, we consistently experienced difficulties in obtaining a sufficient amount of RNA, with the quality necessary to conduct successful RNAseq. After four attempts we collected the required human RNA for RNAseq but we were not able to collect sufficient murine RNA of the necessary quality. However, murine RNA of sufficient quality and amount to conduct quantitative PCR, was collected; therefore, we analyzed the human samples then used quantitative PCR to investigate the murine samples for any differences observed between infected and controls human samples. We also conducted quantitative PCR on human samples to confirm the RNAseq results. Although funding has ended for this project, as of this date, we are continuing to confirm the RNAseq data by quantitative PCR using internal funds. Additionally, during the course of this work, our laboratory published two additional hantavirus papers and one pDC review article that acknowledged the support of our laboratory by this award. Those papers are attached to this report in the section: Publications, Abstracts, and Presentations. The publications pertaining to the pDC-RNAseq analysis will be submitted as an amendment to this report with the next three months. The following sections articulated the data thus far.

Transcriptome analysis of human and murine pDCs suggests that hantavirus infection preferentially promotes Granzyme B upregulation in human pDCs. In this study, all work with infectious virus was conducted in a BSL-3 facility, in compliance with a Memorandum of Understanding with the University of Nevada, Reno, as well as according to NIH, CDC, and OSHA standards. All virus stocks were propagated on

Vero E6 cells. Human pDCs were purified from whole blood using a combination of positive and negative selection (Diamond Plasmacytoid Dendritic Cell Isolation Kit II, Miltenyi Biotec) and murine pDCs were purified from mouse spleens by negative selection (Plasmacytoid Dendritic Cell Isolation Kit, Miltenyi Biotec). Isolated pDCs (>95% purity) were added to 6-well culture plates in complete RPMI media supplemented with IL-3 (10 ng/mL, Peprotech) and allowed to rest overnight. The following day pDCs were infected with either Andes virus strain 23 (ANDV), Hantaan virus strain 76-118 (HTNV) or Prospect Hill virus (PHV). Infections were conducted by incubating each respective virus with pDCs for 1 h at 37 °C, 5% CO^2 at a multiplicity of infection of 0.1. The cells were centrifuged, the virus aspirated and new medium was added and the incubation continued for 24 hours. A mock infection was used as a negative control and all infections were done in duplicate. After 24 hours, the cell cultures were collected using TRIZOL reagent and RNA extracted according to the manufactures instructions.

For NGS analysis, 10 µL of purified total RNA was used to prepare each library. Each respective library was prepared using the Ion total RNAseq kit (Life Technology). Briefly, mRNA was fragmented using RNAse III and purified by nucleic acid binding magnetic beads. The RNA fragments were then hybridized and ligated with adaptor. The ligation product was reverse transcribed to generate cDNA. Purified cDNA was then amplified, during which time each individual strand was barcoded. At the end of amplification, barcoded cDNA was purified and processed for the Ion P1 Chip on the Ion OneTouch system. Final sequencing was conducted using the Ion Proton NGS system. In total, the output of six Ion Proton PI chips generated an average of 12.4 gigabases (Gba) of sequence data per chip. To maximize high-quality alignments for read alignment, gene identification, and quantification, the raw sequence reads were trimmed to eliminate adapters and low-quality reads and short sequences were removed, yielding 477 million trimmed sequences, with an average sequencing depth of 79.6 million independent sequence reads per sample and a mean length of 155 nucleotides (nt) per read. Cleansed reads were successfully aligned to the human genome (GRCh38), using Bowtie2 software [5]. Genomic alignments were then mapped to their respective loci with the coding regions of NCBI RefSeq transcript models to quantitatively measure global gene expression. For differential expression testing, the FPKM (Fragments Per Kilobase of exon per Million fragments mapped) quantities were then transformed into pseudo-count approximations by a simple transformation using gene lengths and effective library sizes. Expressed as counts per million (CPM), a baseline filter was applied to select genes for which CPM>1 (A CPM value of 1 corresponded to a count of approximately 10 in this study). This filtering was performed to extract those genes expressed on one of four samples. The count data of these 33,911 genes were then normalized using upper quartile normalization. Upon normalization, the genes were subjected to a general linear model hypothesis test to determine whether differences between cohorts were statistically significant.

For quantitative PCR analysis, an aliquot of total RNA (40 ng) was used to generate cDNA using the Superscript kit (Invitrogen). Amplification product was used for quantitative PCR (qPCR) analysis using Cybergreen real time PCR mix (Thermofisher) on a BioRad CFX96 qPCR System using the primers sets given in Table 1. All real time PCR values were normalized to that of the GAPDH RNA values in corresponding sample. Normalized GZMB and TNF-A-a values for each virus were adjusted to the corresponding S segment RNA values.

Table 1

Species	Gene	Forward primer	Reverse primer
Human	GZMB	CCCTCAGGCTACCTAGCAAC	TTCGATCTTCCTGCACTGTC
	TNF-Aa	CCTCTTCTCCTTCCTGATCG	ATCACTCCAAAGTGCAGCAG
	GPDH	CATCAATGGAAATCCCATCA	TTCTCCATGGTGGTGAAGAC
Mouse	GZMB	CTGGCTTCATGTCCATTCAC	GGAGAGGGCAAACTTCCATA
	TNF-Aa	CTAGCCAGGAGGGAGAACAG	GCTTTCTGTGCTCATGGTGT
	GAPDH	AGAATTGACAAACGGGACCT	GGAGGAGCAGAGAGCTTGAC
Hantaviruses	ANDV	GCTTTCTGTGCTCATGGTGT	CCTGGTCCCTTTGTTGTCTT
	HTNV	TTGCTGAACTTGGAGCATTC	GAACGATGATTTCTTTCGCA
	PHV	AACTTGCTGCCTCAGGAGAT	CCATCCCTGCAATGTAGATG

Our RNAseq data identified numerous transcriptional differences that need to be confirmed by quantitative PCR, and currently, we are systematically confirming each parameter that is related to our central hypothesis. However, because of the numerous delays and given that the project funding has ended, we are completing this task using internal funds. It is anticipated that this will take two additional months, and one more month to prepare a manuscript of the results.

Two significant discoveries have resulted from this work. First, our ongoing analysis has confirmed the major premise of this project. That is, our data support the premise that human GZMB is upregulated by hantavirus infection (Figure 1). Additionally, we observe that, in contrast to human pDCs, murine pDCs actually have decreased GZMB expression upon hantavirus infection (Figure 2). Additionally, in human pDCs, ANDV strain, which is associated with the greatest pathology, shows the largest increase, followed by HTNV and then PHV (Figure 1) suggesting that the pathogenicity of hantaviruses in human samples is proportional to GZMB expression. In contrast, GZMB is suppressed in murine pDCs upon hantavirus infection with little difference between the three strains (Figure 2).

Quantative PCR suggests that human and murine pDCs support a productive hantavirus infection. Our data also confirm that human pDCs (Figure 3) and murine pDCs (Figure 4) are susceptible to hantavirus infection and also support virus replication; an observation that is previously unreported. Our data further suggest that infection of murine pDCs yields a more productive infection when compared to human pDCs (Figure 3 and 4, respectively) suggesting that the lack of GZMB expression in murine pDCs cannot be attributed to differences in virus replication. Additionally, we observed that Andes virus yields the greatest viral replication; followed by Prospect Hill and then Hantaan, also suggesting that the lack of pathogenicity of observed with Prospect Hill virus cannot be attributed to virus titers alone.

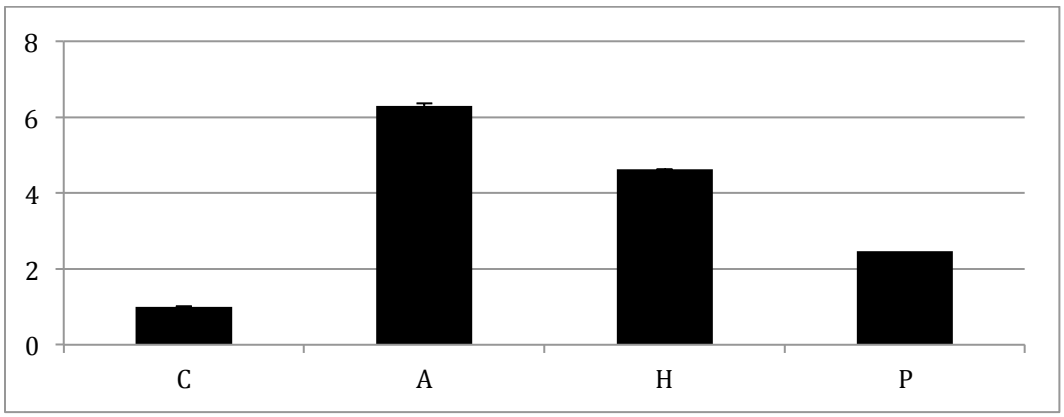

Figure 1. Quantitative PCR of GZMB expression in infected human pDCs,.

C = mock-infected
A = Andes virus
H = Hantaan virus
P = Prospect Hill virus

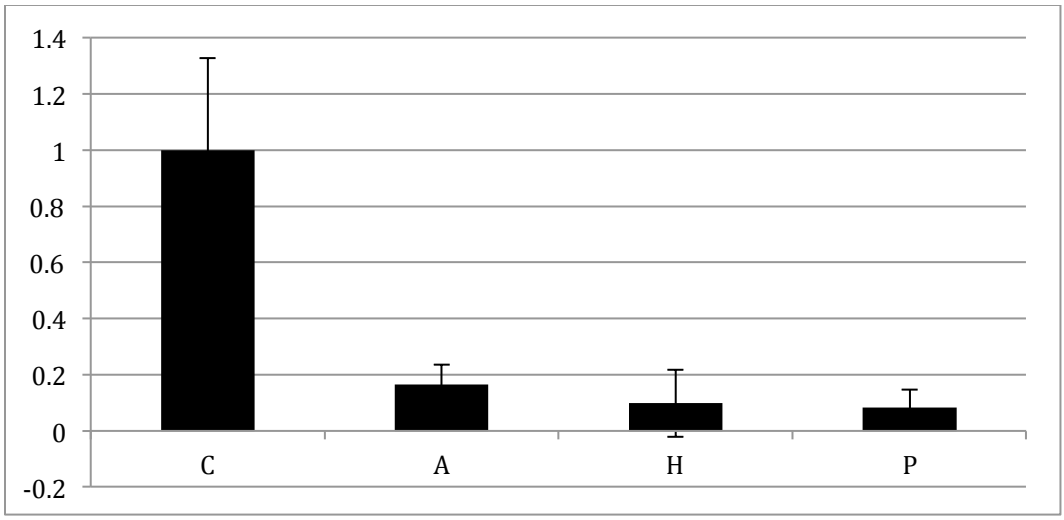

Figure 2. Quantitative PCR of GZMB expression in infected murine pDCs.

C = mock-infected
A = Andes virus
H = Hantaan virus
P = Prospect Hill virus

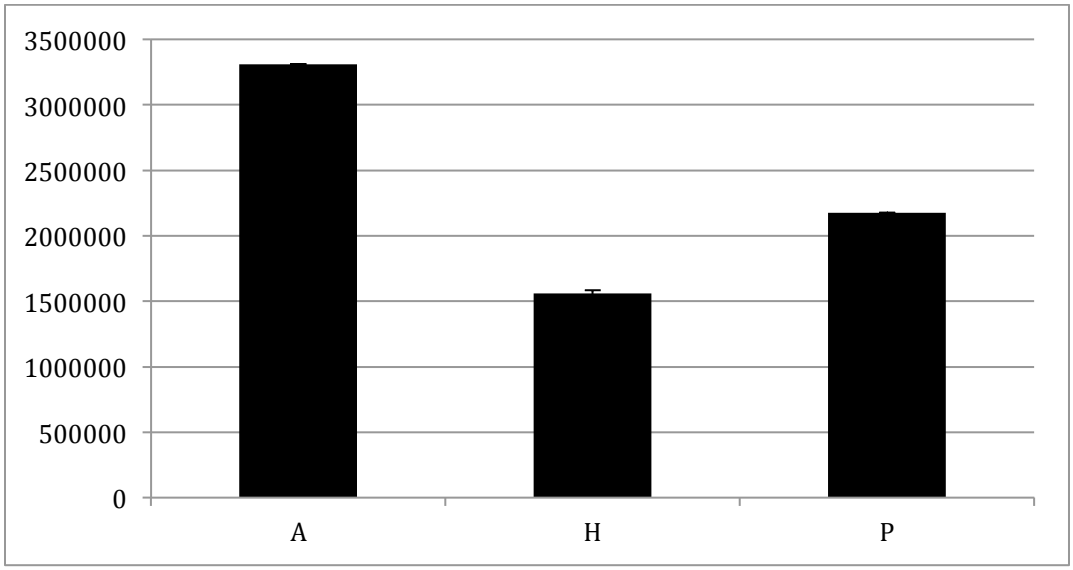

Figure 3. Quantitative PCR of hantavirus replication in human pDCs.

A = Andes virus
H = Hantaan virus
P = Prospect Hill virus
.

Previously, it has been reported that significantly elevated plasma levels of TNF-α, are detected in the acute phase of hantavirus disease, and that the levels correlate with severity of disease [6, 7]. However, in spite of the fact that pDCs are known to produce TNF-α upon TLR engagement [8] we observed no upregulation of TNF-α in hantavirus-infected human pDCs (Figure 5). Similar results were observed upon hantavirus infection of murine pDCs (Figure 6), with the exception of hantaan virus; however, large variation in our replicates suggest that the expression of TNF-α by hantaan-infected pDCs may have been anomalous. We have previously reported that hantavirus-infected HUVECs also do not produce TNF-α, suggesting that the circulating levels commonly observed during hantavirus infection are not the result of pDCs.

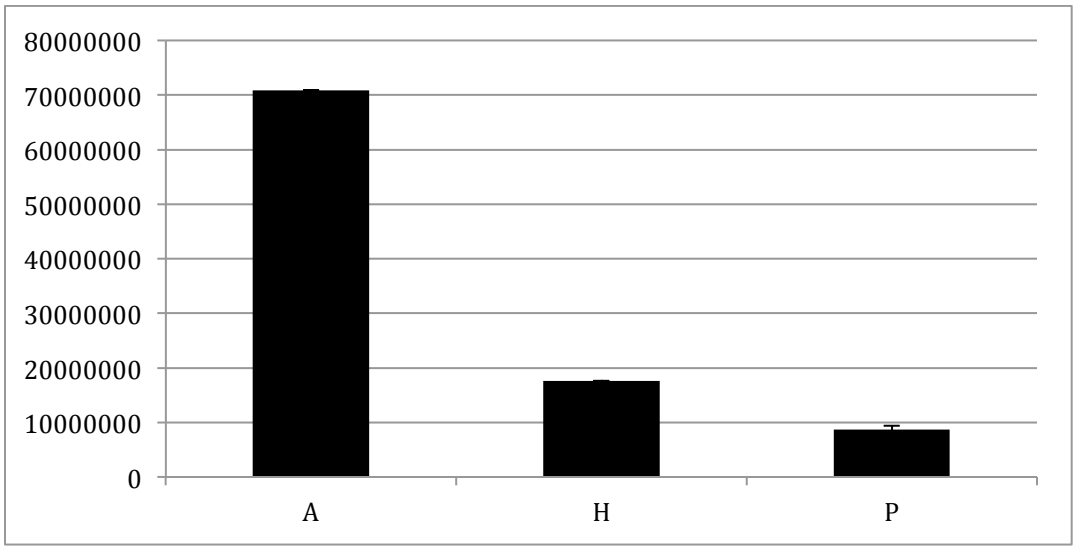

Figure 4. Quantitative PCR of hantavirus replication in murine pDCs.

A = Andes virus
H = Hantaan virus
P = Prospect Hill virus

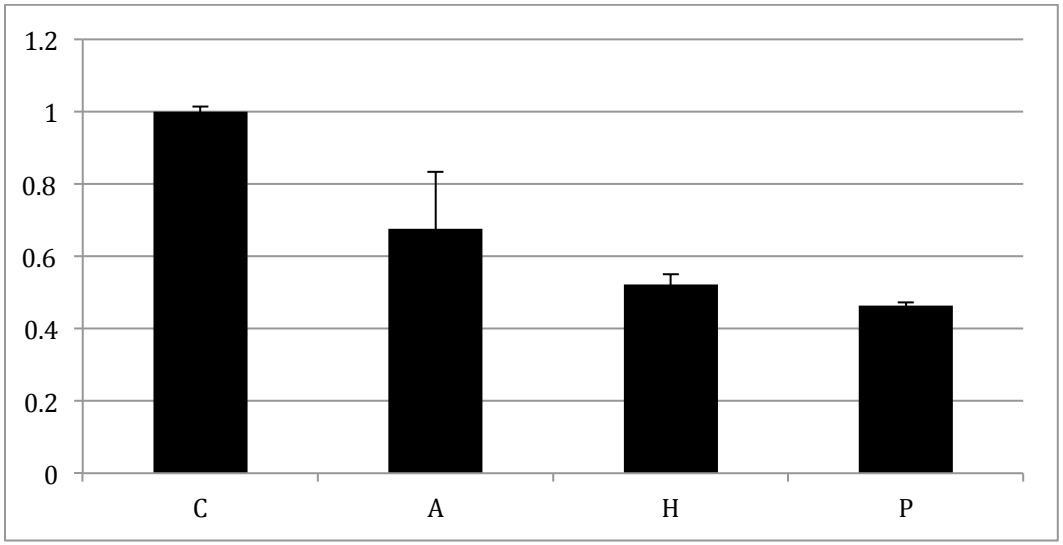

Figure 5. Quantitative PCR of TNF-A-α expression in infected human pDCs.

C = mock-infected
A = Andes virus
H = Hantaan virus
P = Prospect Hill virus

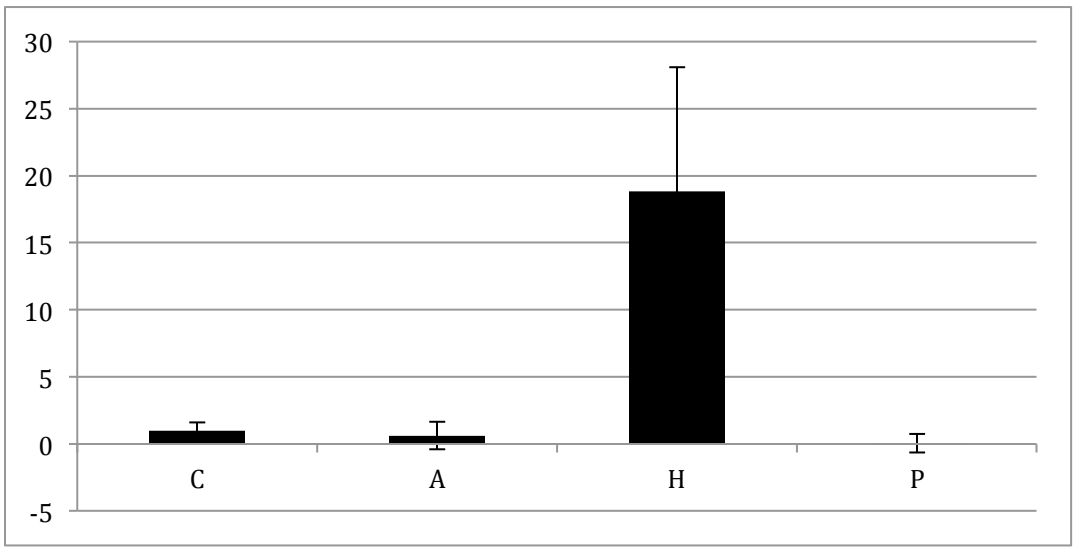

Figure 6. Quantitative PCR of TNF-A-α expression in infected human pDCs.

C = mock-infected
A = Andes virus
H = Hantaan virus
P = Prospect Hill virus

Figure 1. Granzyme B expression by quantative PCR. pDCs were purified From whole blood by magnetic separation. Cells were cultured in RPMI in the presence of IL-3

Summary points of this study

Our data suggests that:

1. pDCs are a putative target of hantavirus infection.
2. pDCs support hantavirus replication.
3. GZMB is upregulated in human pDCs upon hantavirus infection but not murine pDCs.
4. GZMB upregulation in human pDCs correlates with viral pathogenic potential.

4. KEY RESEARCH ACCOMPLISHMENTS:

Because of the difficulties in collect sufficient RNA of the necessary quality to conduct our RNAseq analysis, the completion of this project has been delayed. Consequently, we are only now confirming the RNAseq results by quantitative PCR; however, once this is completed, the results will be published and this report amended.

Nevertheless, the principal premise of this proposal has been confirmed and the key research accomplishments thus far are as follows:

1. **We have completed RNAseq of human and murine infected pDCs.**
2. **We have completed bioinformatical analysis of the RNAseq data.**
3. **We have completed statistical analysis of the RNAseq data.**
4. **We have confirmed the GZMB, TNF-α and viral replication by quantitative PCR.**
5. **We have also published three additional hantavirus papers that acknowledge the support of this award.**

5. CONCLUSION:

The overreaching hypothesis of this proposal is supported by our data. Specifically, human and murine pDCs are susceptible to hantavirus infection; however, only the human pDC is significantly upregulate GZMB. Furthermore, the effect is strain specific, with the most pathogenic strain Andes virus, producing the greatest effect, followed by Hantaan, and then Prospect Hill, the least pathogenic of the three. Numerous other genes in pDCs are affected upon hantavirus infection and there genes are systematically being confirmed.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Hantavirus infection suppresses thrombospondin-1 expression in cultured endothelial cells in a strain-specific manner Front. Microbiol. 2016 July 19; 7:1077.

Multiplex analysis of serum cytokines in humans with hantavirus pulmonary syndrome Front. Immunol., Front Immunol. 2015 Aug 31;6:432.

Plasmacytoid dendritic cells, a role in neoplastic prevention and progression. Eur J Clin Invest. 2015 Jan;45 Suppl 1:1-8.

7. INVENTIONS, PATENTS AND LICENSES

N/A

8. REPORTABLE OUTCOMES

N/A

9. OTHER ACHIEVEMENTS

During the course of our work with pDCs, we also included infections of human umbilical vein endothelial cells (HUVEC), which is the classic research tool for hantavirus work. Because of the high-risk nature of the present pDC project, we felt that it was prudent to include HUVEC infections, as a fail-safe, to ensure that the project would be guaranteed to yield publishable results. Althought RNA arrays have been conducted for hantaviruses [9], to the best of our knowledge, RNAseq analysis has never been reported for hantavirus infection of HUVECs. By investigating the pathogenic and non-pathogenic strains, we would potentially identify specific targets that may be confirmed in the infected pDCs but would also stand on its own as novell research data. Because present study provided access into the BSL-3 facility, we also included the work with HUVECs. The RNAseq analysis of HUVECs was made possible through a donation may to us by the UNR genomics facility and Illumina and the HUVEC cells were already on hand. The HUVECs were cultured and infected simultaneously with the pDCs using the same experimental conditions, including the two pathogenic and one non-pathogenic hantavirus strain. Once we have completed the quantitative PCR confirmation of the pDC-RNAseq results, we will commence with the analysis of the HUVEC data. The pDC RNAseq was conducted

using the Life Technologies Ion Proton platform; however, the HUVEC RNAseq was conducted on an Illumina platform, therefore, we feel that these two studies will need to be published separately, but both studies will acknowledge present award as a source of funding.

10. REFERENCES

1. **Warner, G.S., *Hantavirus illness in humans: review and update.* South Med J, 1996. 89(3): p. 264-71.**

2. **Botten, J., et al., *Experimental infection model for Sin Nombre hantavirus in the deer mouse (Peromyscus maniculatus).* Proc Natl Acad Sci U S A, 2000. 97(19): p. 10578-83.**

3. **Botten, J., et al., *Persistent Sin Nombre virus infection in the deer mouse (Peromyscus maniculatus) model: sites of replication and strand-specific expression.* J Virol, 2003. 77(2): p. 1540-50.**

4. **Shodell, M. and F.P. Siegal, *Circulating, interferon-producing plasmacytoid dendritic cells decline during human ageing.* Scand J Immunol, 2002. 56(5): p. 518-21.**

5. **Langmead, B. and S.L. Salzberg, *Fast gapped-read alignment with Bowtie 2.* Nat Methods, 2012. 9(4): p. 357-9.**

6. **Terajima, M., et al., *Immune responses to Puumala virus infection and the pathogenesis of nephropathia epidemica.* Microbes Infect, 2004. 6(2): p. 238-45.**

7. **Settergren, B., et al., *Pathogenetic and clinical aspects of the renal involvement in hemorrhagic fever with renal syndrome.* Ren Fail, 1997. 19(1): p. 1-14.**

8. **Gibson, S.J., et al., *Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod.* Cell Immunol, 2002. 218(1-2): p. 74-86.**

9. **Geimonen, E., et al., *Pathogenic and nonpathogenic hantaviruses differentially regulate endothelial cell responses.* Proc Natl Acad Sci U S A, 2002. 99(21): p. 13837-42.**

11. Appendices

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 July 2016
doi: 10.3389/fmicb.2016.01077

Hantavirus Infection Suppresses Thrombospondin-1 Expression in Cultured Endothelial Cells in a Strain-Specific Manner

Svetlana F. Khaiboullina[1,2], Sergey P. Morzunov[3], Stephen C. St. Jeor[4], Albert A. Rizvanov[2] and Vincent C. Lombardi[1,5]**

[1] Nevada Center for Biomedical Research, Reno, NV, USA, [2] Department of Genetics, Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia, [3] Nevada State Public Health Laboratory, University of Nevada, Reno School of Medicine, Reno, NV, USA, [4] Department of Immunology and Microbiology, University of Nevada, Reno School of Medicine, Reno, NV, USA, [5] Department of Biochemistry and Molecular Biology, University of Nevada, Reno, NV, USA

OPEN ACCESS

Edited by:
Fabrice Merien, Auckland University of Technology, New Zealand

Reviewed by:
Xiaodong Zhang, Jilin University, China
Bruno Pozzetto, Jean Monnet University, France

***Correspondence:**
Albert A. Rizvanov
Albert.Rizvanov@kpfu.ru
Vincent C. Lombardi
vlombardi@medicine.nevada.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *06 November 2015*
Accepted: *27 June 2016*
Published: *19 July 2016*

Citation:
Khaiboullina SF, Morzunov SP, St. Jeor SC, Rizvanov AA and Lombardi VC (2016) Hantavirus Infection Suppresses Thrombospondin-1 Expression in Cultured Endothelial Cells in a Strain-Specific Manner. Front. Microbiol. 7:1077. doi: 10.3389/fmicb.2016.01077

Hantavirus infection is associated with two frequently fatal diseases in humans: Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). The pathogenesis of hantavirus infection is complex and not fully understood; however, it is believed to involve virus-induced hyperinflammatory immune responses. Thrombospondin-1 (THBS1) is a large homotrimeric protein that plays a putative role in regulating blood homeostasis. Hyperresponsiveness to inflammatory stimuli has also been associated with defects in the THBS1 gene. Our data suggest that hantavirus infection of human umbilical cord vein endothelial cells (HUVEC) suppress the accumulation of THBS1 in the extracellular matrix. Additionally, this suppression is dependent on virus replication, implying a direct mechanism of action. Our data also imply that the pathogenic Andes and Hantaan strains inhibit THBS1 expression while the non-pathogenic Prospect Hill strain showed little inhibition. These observations suggest that a dysregulation of THBS1 may contribute to the pathogenesis of hantavirus infection.

Keywords: coagulation, HPS, HFRS, THBS1, TSP1, Prospect Hill, Hantaan, Andes

INTRODUCTION

Hantaviruses are negative strand RNA viruses belonging to the family *Bunyaviridae* (Schmaljohn and Nichol, 2001; Schmaljohn, 2007). They are the etiological agent associated with two frequently fatal human illnesses: Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS; Jenison et al., 1995; Enria et al., 2001; Linderholm and Elgh, 2001; Peters and Khan, 2002). Postmortem analyses of HFRS and HPS cases typically reveal high viral loads in endothelial cells, suggesting that the vascular endothelium is a principal site of infection (Zaki et al., 1995). *In vitro* studies have confirmed that human vascular endothelial cells are susceptible to hantavirus infection; however, infection does not produce a visible cytopathic effect (Pensiero et al., 1992; Khaiboullina et al., 2004).

HFRS and HPS are characterized by high fever and myalgia; however, kidney failure and bleeding are typical of HRFS while HPS manifests with pneumonia and cardiovascular dysfunction (Kanerva et al., 1998; Enria et al., 2001; Linderholm and Elgh, 2001). Despite different clinical

presentations, decreased platelet counts, and changes in blood chemistry are commonly observed during the early phase of both syndromes (Kanerva et al., 1998; Peters et al., 1999). Disturbed hemostasis is a hallmark of HFRS (Linderholm and Elgh, 2001; Sundberg et al., 2011) as is progressive disseminated intravascular coagulation (DIC) syndrome (Connolly-Andersen et al., 2015); however, severe shock and renal failure tend to associate with more severe illness (Lee et al., 1989; Rasche et al., 2004; Sundberg et al., 2011). HPS commonly manifests with pneumonia and severe cardiovascular dysfunction as a result of progressive leakage of fluid from the blood into the lungs, resulting in pulmonary edema, hypoxia, and circulatory collapse (Enria et al., 2001). Although DIC is not the most prevalent pathology during HPS, laboratory, and histological examinations have revealed the presence of all symptoms of DIC (Zaki et al., 1995). DIC is an acquired disorder in which the proteins that control blood clotting become constitutively activated, resulting in consumption of platelets and conversion of fibrinogen to fibrin (Slofstra et al., 2003). With blood platelets and clotting factors exhausted, internal, and external bleeding typically ensues (Peters et al., 1999). In the normal steady state, endothelial cells control blood homeostasis by continuously expressing several proteins (Cirino et al., 2000; Dugina et al., 2002), many of which interact with thrombospondin-1 (THBS1); a large trimeric glycoprotein secreted by several cell types including endothelial cells, and activated platelets (McPherson et al., 1981; Mast et al., 2000; Resovi et al., 2014; Prakash et al., 2015).

Once secreted, THBS1 suppresses proliferation and migration of endothelial cells, as well as signals their apoptosis (Khan et al., 1996; Lawler and Detmar, 2004). Additionally, THBS1 can induce endothelial cell actin reorganization and focal adhesion disassembly (Greenwood et al., 1998; Goicoechea et al., 2000). Several proteases involved in angiogenesis and endothelial integrity, such as urokinase, plasminogen, matrix metalloproteinase, thrombin, cathepsin, and elastase are also influenced by THSB1 (Hogg et al., 1993; Albo et al., 1997; Bein and Simons, 2000; Liu et al., 2009; Yang et al., 2012; Zhao et al., 2015). Furthermore, THBS1 can directly affect the activity of plasmin and urokinase plasminogen activator, which are important for degradation of fibrin (Silverstein et al., 1986; Hogg et al., 1992; Rabhi-Sabile et al., 1998). Finally, THBS1 controls von Willebrand (vW) factor multimer sizes by reduction of disulfide bonds, which link the vW factor subunits together (Xie et al., 2001). Secretion of THBS1 by endothelial cells can be modulated by proinflammatory cytokines such as TNFα and IL-1, two cytokines that are commonly observed in association with hantavirus infection (McPherson et al., 1981; Lawler and Detmar, 2004; Lopez-Dee et al., 2011). Indeed, several proteins modulated by THBS1 have been implicated in hantavirus pathology. For instance, Bondu and coworkers observed elevated levels of thrombin in severe HPS cases (Saumet et al., 2002; Bondu et al., 2015) and Sadeghi et al. observed TGFB1 to be elevated during the late phase of Puumala hantavirus infection (Sadeghi et al., 2011; Chu et al., 2013). Also, Strandin and colleagues reported that tissue plasminogen activator was strongly upregulated in severe cases of Puumala hantivirus infection (Strandin et al., 2016). Additionally, Davidovich et al. reported a three-fold increase in serum vW in HRFS patients during their oliguric phase (Davidovich et al., 1993). Although the molecular mechanisms involved in hantavirus pathology are not well-understood it is reasonable that THBS1 may play a role in the pathophysiology of hantavirus infection through the interactions with the abovementioned proteins.

Recently, decreased levels of serum THBS1 have been reported in association with HFRS (Liu et al., 2008; Laine et al., 2014). Liu et al. suggested that insufficient production or increased consumption of THBS1 contribute to the impaired integrity of small capillaries in subjects with HFRS (Liu et al., 2008). In the present study, we demonstrate that *in vitro* hantavirus infection of endothelial cells suppresses transcription of THBS1 and its subsequent accumulation in the extracellular matrix. This suppression is dependent on viral replication, but is independent of IL-6 and CCL5 expression by infected cells. Additionally, these observations were strain-specific with the pathogenic Andes (ANDV) and Hantaan (HTNV) strains suppressing THBS1 expression while non-pathogenic Prospect Hill (PHV) strain showed little inhibition.

METHODS

Human umbilical cord vein endothelial cells (HUVEC) and Vero clone E6 (Vero E6) were obtained from Lonza, Inc. (Portsmouth, NH) and American Type Culture Collection (ATCC, Manassas, VA), respectively. HUVEC were grown in MCDB 131 medium, supplemented with human vascular endothelial cell growth factor, hydrocortisone, 2% fetal bovine serum (FBS), human fibroblast growth factor (0.5 mL; 1 μg/mL), ascorbic acid, heparin (0.5 mL; 1 μg/mL), and gentamicin. Cells were used at passages 2–4. Vero E6 cells were grown in DMEM medium containing 20% FBS and gentamicin.

All work with infectious virus was conducted in a BSL-3 facility, in compliance with a Memorandum of Understanding with the University of Nevada, Reno, as well as according to NIH, CDC, and OSHA standards. All virus stocks were propagated on Vero E6 cells. Andes virus strain 23 (ANDV) and Hantaan virus strain 76–118 (HTNV) were a generous gift from Dr. Thomas Ksiazek (CDC, Atlanta, GA), and Prospect Hill virus (PHV) was a generous gift from Dr. Connie Schmaljohn (Fort Detrick, MD). In all experiments, cells were infected at a virus to cell ratio of 3 (MOI of 3) except where indicated. In some experiments, the virus was inactivated with 2×10^6 rads of gamma radiation. Infections were conducted by incubating each respective virus on HUVEC for 1 h at 37°C, 5% CO_2. The cell monolayers were then washed with HBBS, and new medium was added and the incubation continued for the times indicated. For some experiments, new culture medium was supplemented with 10 ng/mL TNFα, 10 ng/mL TGFβ, 50 ng/ml IL-6, or 12 ng/mL CCL5 (R&D Systems). Mock-infected cells and/or untreated cells were used as controls. All experiments were done in duplicate.

Immunofluorescence

Hantavirus-infected and mock-infected HUVEC monolayers were fixed with methanol/acetone (3:1), washed three times with

PBS (pH 7.4), and incubated with glycine buffer (10 mM glycine in PBS pH 7.4) for 30 min at room temp. Slides were again washed three times with PBS and permeabilized with Triton-X 100 solution (0.1% in PBS pH 7.4) for 30 min at room temp. Slides were next washed three times with PBS and incubated for 30 min at room temp with an appropriate combination of antibodies (summarized in **Table 1**). Slides were washed again three times in PBS, and incubated with the respective antibody combinations for 30 min at room temp in the dark. Images were captured using a Nikon C1 fluorescent microscope with Easy C1 software.

Western Blot

Hantavirus-infected and mock-infected HUVEC were lysed in 200 μL of 0.1% sodium dodecyl sulfate (SDS) solution and standardized using the Better Bradford™ Assay Kit (Pierce, Div. of Thermo Fisher, Waltham, MA). Proteins were electroblotted for 15 min, at 4 mA/cm^2 onto PVDF membranes (Bio-Rad, Hercules, CA) and blocked for 1 h at room temp with blocking buffer (5% non-fat dry milk in PBS, pH 7.4 and 0.5% Tween 20). Membranes were then washed three times in washing buffer (PBS pH 7.4 and 0.5% Tween 20) and incubated with the respective antibodies in blocking buffer for 12 h at 4°C. Antigen–antibody complexes were identified with goat anti-rabbit-HRP or goat anti-mouse-HRP conjugated antibodies (1:1000 in blocking buffer) and developed using insoluble HRP substrate (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. Western blots were quantitatively measured using Scion Image software version beta 4.0.2.

Real Time PCR

Hantavirus-infected HUVEC and mock-infected cells were collected at selected time points and total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. To conduct first strand cDNA synthesis, 3 μL RNA (1 μg total), 1 μL random primer mix (50 μM), and 8.5 μL RNase-free water were combined, denatured at 70°C for 10 min, and chilled at 4°C to anneal the primers. cDNA was then synthesized by adding 1 μL of each dNTP (10 mM; GIBCO, Division of Life Technologies), 1x RT buffer (Promega, Madison, WI), 200 U of MMLV reverse transcriptase (Promega), and 20 U of rRNasin RNAse inhibitor (Promega) in a 20 μL total reaction volume. After incubation for 10 min at 25°C, the reaction proceeded for 1 h at 42°C and was terminated by heating at 95°C for 5 min. cDNA was stored at −20°C until analyzed.

TaqMan analyses were performed using TaqMan minor groove binding probes on an ABI Prism 7000 Sequence Detection System. Each PCR reaction (25 μL) consisted of 1 μL of cDNA, 1õ Platinum qPCR Supermix-UDG (Life Technologies), 200 nM of each primer, and 100 nM of probe. The cDNA of each respective sample was diluted 1:1000 with nuclease free water before TaqMan analyses. The 18S ribosomal gene was used as an endogenous control for all analyses. Standard curves for relative quantification of viral S segment RNA, cellular genes, and 18S RNA were created using serial dilutions of cDNA from infected and uninfected control samples depending on specifics of the experimental design. All TaqMan reactions were performed in triplicate. In TaqMan experiments, TaqMan values of cellular gene mRNA were normalized against TaqMan values for the 18S gene of a corresponding sample. For some experiments, normalized values were expressed as relative values to the same gene expressed in the corresponding control group. The sequences of primers and probe for THBS1 are summarized in **Table 2**.

Statistical Analysis

Data are presented as mean ± *SE*. Statistical analyses were performed using Student's *t*-test for comparisons between individual experimental groups (infected and non-infected). Significance was established at a value of $p < 0.01$.

RESULTS

Effect of ANDV, HTNV, and PHV Infection and Cytokine Treatment on THBS1 Accumulation in the Extracellular Matrix of HUVEC

A central dogma of hantavirus pathogenesis is that virus replication leads to endothelial cell dysregulation (Connolly-Andersen et al., 2014). THBS1 is an extracellular matrix protein

TABLE 1 | Antibodies.

Antibody	Concentration	Supplier
Mouse anti-THBS1 mAb	1:400	Sigma
Mouse anti-PUU* mAb	1:100	Dr. Thomas Ksiazek (CDC)
Mouse anti-p-c-Jun mAb	1:100	Santa-Cruz
Rabbit anti-NF-kB	1:100	Santa-Cruz
Rabbit anti-N Protein	1:500	In house
Goat anti-mouse-Alexa 488	1:800	Molecular probes
Donkey anti-rabbit-Alexa 555	1:800	Molecular probes

**Hantavirus (Puumala) nucleocapsid protein.*

TABLE 2 | Sequence and position of primers and probes used for TaqMan.

Gene	Forward primer	Reverse primer	TaqMan probe
ANDV*	tcacgccaggacgatttagg (414–433)	ggctttgacctgtgctggaa (472–453)	caattgcttgtggcctt (435–451)
PHV*	ggctgacaaagtaaaggcattc (738–760)	cgtggctcagcctttaggaa (797–778)	tgaccagaaatgtcc (761–776)
HTV*	gcagcagttagcctccttggt (787–807)	tgccgctgccgtaagtagt (845–827)	tcctgcaacaaacagg (810–825)
THBS1	gaggcggcctcccctat (1115–1131)	tcaacagtccattcctcgttatttc (1180–1156)	tatcacaacggagttcagtac (1134–1154)
RANTES	cccgcagaggaagcacaa (858–875)	gcgaagatttcccgtaaactttc (919–897)	actacgcgggctgc (861–875)
IL-6	ctgcgcagctttaaggagttc (513–533)	ccatgctacatttgccgaaga (574–554)	cagtccagcctgaggg (537–552)

**Nucleocapsid "S" segment.*

secreted by endothelial cells and has putative roles in cell adhesion, platelet aggregation, and the regulation of fibrinolysis (Silverstein et al., 1984; Roberts et al., 2010). With this in mind, we sought to determine if hantavirus infection and replication affects THBS1 accumulation in cultured HUVEC. Additionally, we investigated if pathogenic ANDV and HTNV and non-pathogenic PHV differ in their abilities to influence THBS1 accumulation.

HUVEC monolayers infected with ANDV, HTNV, and PHV were fixed and probed by immunohistochemistry (IHC) for THBS1 accumulation in the extracellular matrix (**Figure 1**). In mock-infected cells, THBS1 was equally distributed throughout the extracellular matrix (**Figure 1A**). In contrast, accumulation of THBS1 was greatly reduced in ANDV and HTNV infected HUVEC (**Figures 1B,C**, respectively). In cells infected with PHV, accumulation of THBS1 was observed throughout the extracellular matrix similar to that observed in uninfected control cells (**Figure 1D**).

The interactions between THBS1 and transforming growth factor beta (TGFβ) are complex. TGFβ can regulate the expression of extracellular matrix proteins, including THBS1 (Negoescu et al., 1995; Nakagawa et al., 2005). Conversely, discrete sequences of THBS1 have been shown to activate TGFβ (Schultz-Cherry et al., 1995). Therefore, in order to investigate the effect of TGFβ on the accumulation of THBS1 in hantavirus-infected cells we duplicated the above experiment but supplemented the HUVEC culture media with TGFβ. Treatment of mock-infected cells with TGFβ did not significantly affect

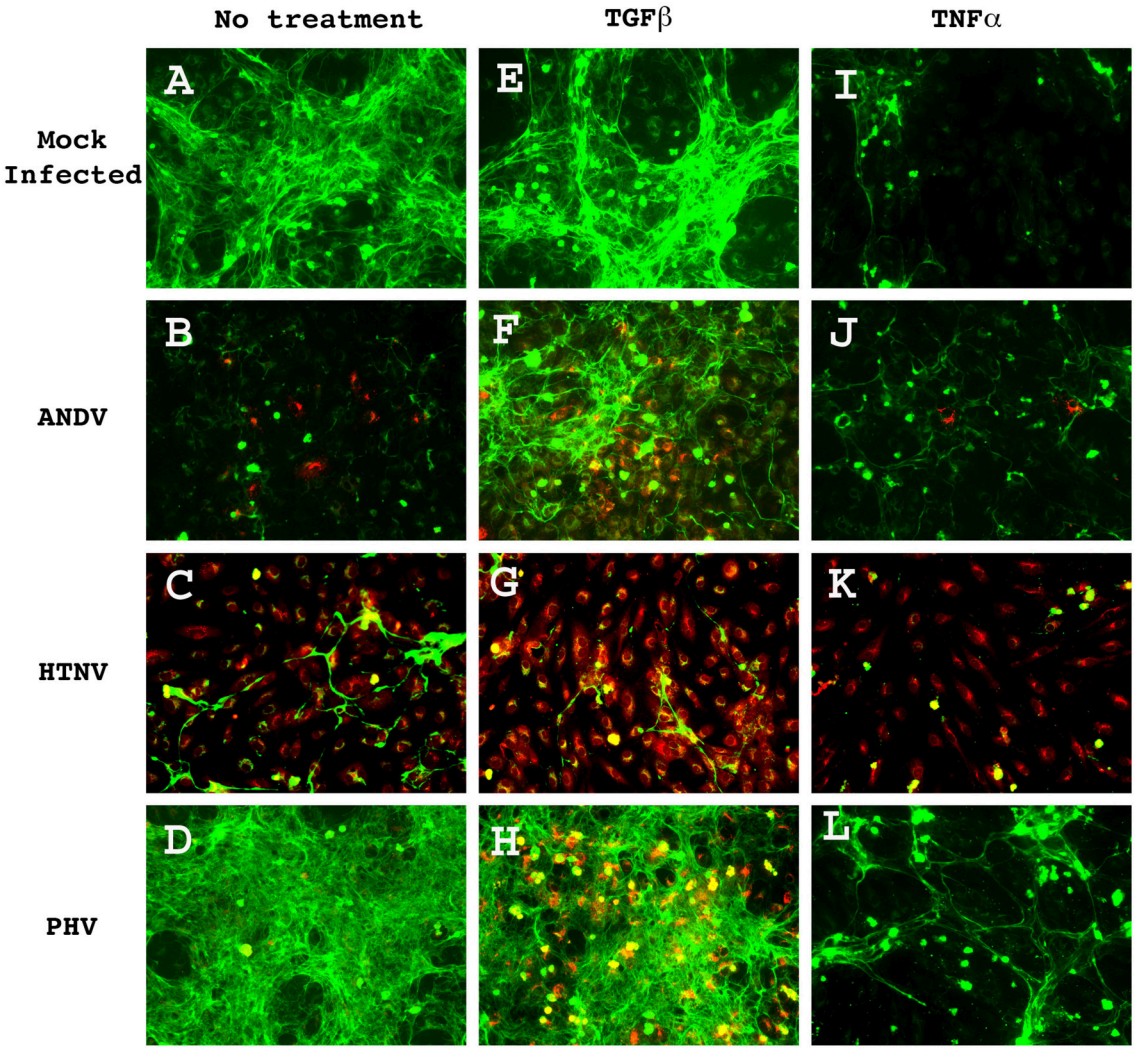

FIGURE 1 | THBS1 accumulation in the extracellular matrix of HUVEC monolayers infected with pathogenic (ANDV and HTNV) and non-pathogenic (PHV) hantavirus in the presence and absence of TGFβ or TNFα. Monolayers were infected at MOI 3, with the indicated strains, treated with 10 ng/mL of TGFβ or TNFα, and fixed at 72 h PI. Monolayers were stained with rabbit anti-hantavirus N protein polyclonal antibody followed by donkey anti-rabbit-Alexa 555 secondary (red) and mouse anti-THBS1 mAb followed by a goat anti-mouse-Alexa 488 secondary (green). **(A)** Mock infected, no treatment; **(B)** ANDV infected, no treatment; **(C)** HTNV infected, no treatment; **(D)** PHV infected, no treatment; **(E)** Mock infected, TGFb treated; **(F)** ANDV infected, TGFb treated; **(G)** HTNV infected, TGFb treated; **(H)** PHV infected, TGFb treated; **(I)** Mock infected, TNFa treated; **(J)** ANDV infected, TNFa treated; **(K)** HTNV infected, TNFa treated; **(L)** PHV infected, TNFa treated.

THBS1 accumulation in the extracellular matrix (**Figure 1E**); however, when ANDV-infected TGFβ treated and non-treated cells were compared, a significant increase in the accumulation of THBS1 was observed in the TGFβ-treated cells (**Figure 1F**). Interestingly, when compared to untreated cells, TGFβ did not substantially affect THBS1 accumulation in the extracellular matrix of cells infected with HTNV and PHV (**Figures 1G,H**, respectively).

Previous reports suggest that secretion of THBS1 by endothelial cells may be modulated by proinflammatory cytokines such as tumor necrosis factor alpha (TNFα; Morandi et al., 1994). To investigate this possibility in the context of hantavirus infection, 10 ng/mL of TNFα was added to the culture media of hantavirus infected, and mock-infected HUVEC. Addition of TNFα reduced THBS1 accumulation in the extracellular matrix of mock-infected HUVEC (**Figure 1I**). THBS1 no longer appeared equally distributed throughout the monolayer as seen in the mock-infected control without TNFα treatment. THBS1 accumulation in the matrix of ANDV and HTNV infected HUVEC remained low after treatment with TNFα (**Figures 1J,K**, respectively). Although PHV infection did not affect accumulation of THBS1 in the extracellular matrix of untreated cells, TNFα treatment did inhibited THBS1 accumulation in PHV infected cells similar to that of uninfected controls (**Figure 1L**).

Transcriptional Activation of THBS1 in Hantavirus Infected HUVEC

The effects of hantavirus infection and TNFα treatment on THBS1 transcription were analyzed using TaqMan (**Figure 2**). Untreated and TNFα-treated (10 ng/mL) HUVEC were infected with ANDV, HTNV, PHV, or mock-infected and total RNA was collected at 12, 24, and 72 h post infection (PI). Pathogenic ANDV and HTNV showed significantly reduced transcriptional activity of THBS1 in HUVEC 12 h PI when compared to mock-infected cells ($p < 0.01$; **Figures 2.2**, **2.3**). Transcription of THBS1 RNA in ANDV and HTNV infected cells returned to the levels of mock-infected cells at 72 h PI (**Figures 2.2**, **2.3**). In HUVEC infected with non-pathogenic PHV, THBS1 RNA levels did not differ significantly from that of mock-infected HUVEC at all time points PI (**Figure 2.4**). TNFα treatment significantly suppressed THBS1 RNA levels in mock-infected HUVEC at 12, 24, and 72 h PI compared to untreated cells ($p < 0.01$; **Figure 2.5**). THBS1 RNA levels were reduced in TNFα-treated HUVEC infected with pathogenic ANDV and HTNV similar to that of uninfected TNFα-treated controls at all time points ($p < 0.01$; **Figures 2.6**, **2.7**). Interestingly, THBS1 RNA levels in PHV-infected HUVEC treated with TNFα did not differ significantly from that of PHV-infected cells without TNFα treatment at 12 h PI (**Figures 2.4**, **2.8**). However, THBS1 RNA levels were significantly lower in PHV-infected HUVEC treated with TNFα as compared to that in PHV-infected cells without TNFα treatment at 24 and 72 h PI ($p < 0.01$; **Figures 2.4**, **2.8**).

Effect of Hantavirus Replication on THBS1 Accumulation in the Extracellular Matrix of HUVEC

To assess if an active infection is required for the accumulation of THBS1 in the extracellular matrix, HUVEC were inoculated with ANDV and HTNV, as well as the same viruses inactivated by gamma radiation. Total RNA was then collected at selected time points after inoculation (1, 24, 48, and 72 h PI) for evaluation using TaqMan. ANDV and HTNV S segment RNA was measured to confirm hantavirus replication (data not shown). Transcriptional activity of THBS1 in HUVEC inoculated with inactivated ANDV or HTNV did not differ significantly from that of mock-infected HUVEC at all selected time points (1, 24, 48, and 72 h PI; **Figure 3A**). However, the levels of

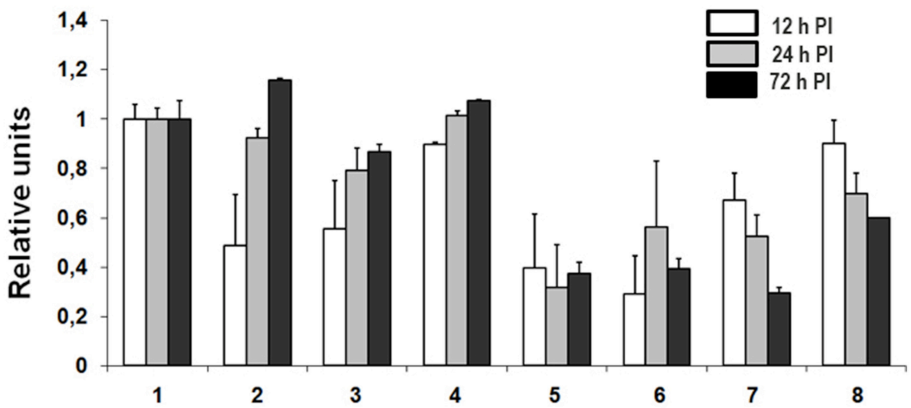

FIGURE 2 | Transcriptional activation of THBS1 in hantavirus infected HUVEC. Cells were infected with pathogenic (ANDV and HTNV) and non-pathogenic (PHV) hantaviruses at MOI 3. TNFα was used at 10 ng/mL where indicated. Total RNA was collected at 12, 24, and 72 h PI and used to determine transcription of THBS1 mRNA by TaqMan analysis. Values of THBS1 mRNA were normalized to the 18S mRNA in the corresponding sample. Relative units of THBS1 mRNA were obtained by dividing normalized THBS1 values of infected cells by those of uninfected controls. Each experiment was performed three times and each TaqMan reaction was performed in duplicate. **(1)** Uninfected, **(2)** ANDV infected, **(3)** HTNV infected, **(4)** PHV infected, **(5)** TNFα treated, **(6)** TNFα treated and ANDV infected, **(7)** TNFα treated and HTNV infected, **(8)** TNFα treated and PHV infected.

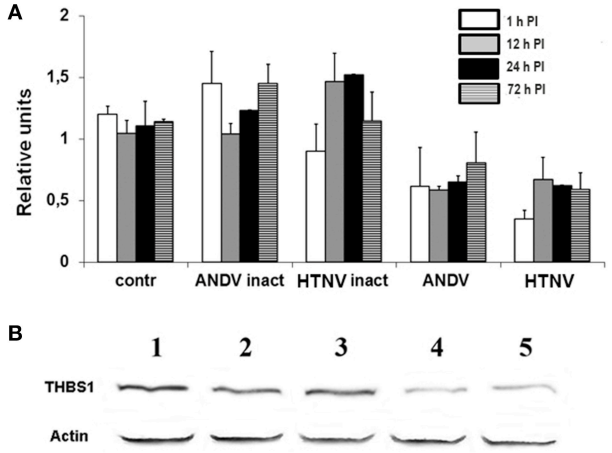

FIGURE 3 | Effect of hantavirus replication on THBS1 transcriptional activity and protein expression in HUVEC. Cells were inoculated with gamma radiation inactivated or infectious ANDV and HTNV at MOI of 1. Total RNA was collected and used to evaluate THBS1 transcription **(A)**. Values of THBS1 were normalized to the corresponding values of 18S RNA. Relative units were calculated by dividing normalized values in infected group by normalized values in mock-infected control group. To determine the effect of hantavirus replication on THBS1 protein accumulation in HUVEC **(B)**, Total proteins collected from HUVEC inoculated with gamma radiation inactivated and infectious ANDV and HTNV (MOI 1; 72 h PI) were separated by SDS-PAGE and transferred onto the PVDF membrane. Membranes were probed with anti-THBS1 mAb followed by anti-mouse HRP-conjugated secondary antibody. 1. Mock infected, 2. Inactivated ANDV, 3. Inactivated HTNV, 4. Infectious ANDV, and 5. Infectious HTNV.

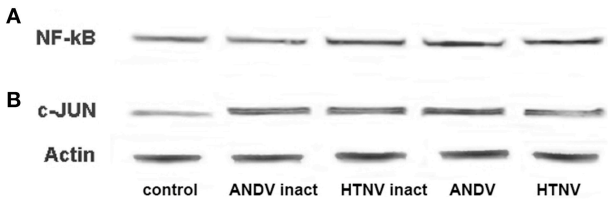

FIGURE 4 | Effect of hantavirus replication on NF-kB expression and c-Jun activation. Total proteins collected from HUVEC inoculated with gamma radiation inactivated and infectious ANDV and HTV (MOI of 1; 72 h PI) were separated in SDS-PAGE and transferred onto the PVDF membrane. Membranes were probed with anti-NF-kB **(A)** or anti-phospho-c-Jun **(B)** mAbs followed by anti-mouse-HRP conjugated secondary antibody.

THBS1 RNA were significantly lower in HUVEC inoculated with competent ANDV or HTNV at each selected time point PI when compared to cells inoculated with inactivated ANDV or HTNV ($p < 0.01$; **Figure 3A**). The effects of competent and gamma-irradiated ANDV and HTNV on the expression of THBS1 in HUVEC were also analyzed using Western blot (**Figure 3B**). THBS1 expression in HUVEC inoculated with gamma-irradiated ANDV or HTNV did not differ from that of mock-infected HUVEC (**Figure 3B**). In contrast, cells infected with competent ANDV or HTNV showed clear reduction in THSB1 expression (**Figure 3B**).

Effect of Hantavirus Replication on c-Jun Activation and NF-kB Expression

Gamma-irradiation hinders virus replication, but preserves viron integrity, thus allowing viral proteins to interact with cellular proteins in the absence of virus replication. Additionally, it has been shown that the expression of THBS1 can be inhibited by activation of the c-Jun member of the AP-1 family of transcription factors (Bohmann et al., 1987; Curran and Franza, 1988), as well as NF-kB activation (Cinatl et al., 2000). With this in mind, we sought to determine if viral replication was required to activate these transcription factors. We observed similar levels of NF-kB expression and c-Jun activation in HUVEC inoculated with inactivated or competent ANDV and HTNV (**Figures 4A,B**, respectively at 72 h PI). These observations suggest NF-kB expression and c-Jun activation are independent of virus replication and, therefore, are unlikely to represent the mechanism of THSB1 inhibition by hantaviruses.

Activation of CCL5 and IL-6 Expression in HUVEC Infected with ANDV, HTNV, and PHV

Hantaviruses may affect the expression of THBS1 in endothelial cells by indirect mechanisms such as by promoting cytokine and chemokine production. At least two cytokines, TNFα and interleukin-1 (IL-1), have been shown to suppress THBS1 expression by endothelial cells (Morandi et al., 1994). However, we as well as others have shown that hantavirus infection does not activate TNFα or IL-1 expression in endothelial cells (Sundstrom et al., 2001; Geimonen et al., 2002; Khaiboullina et al., 2004). Nevertheless, we have previously shown that hantaviruses promote the expression of CCL5 in HUVEC (Khaiboullina et al., 2004). During the course of these previous studies, we did not observe any changes in IL-6 gene expression in hantavirus-infected cells at early time points (3 and 12 h PI; Khaiboullina et al., 2004). We did however observe that the transcriptional regulator nuclear factor for interleukin-6 (CCAAT/Enhancer Binding Protein), which activates IL-6 gene expression (Akira et al., 1990; Kinoshita et al., 1992), was upregulated in infected cells (Geimonen et al., 2002; Khaiboullina et al., 2004) suggesting that IL-6 expression might be activated at a later time point. Based upon these data, we hypothesized that hantavirus-induced CCL5 and IL-6 expression may lead to a change in the accumulation of THBS1 in HUVEC. We therefore infected HUVEC with ANDV, HTNV, and PHV and collected total RNA at selected time points (12, 24, and 72 h PI) for TaqMan analysis and collected cell culture media supernatants for cytokine analysis by ELISA. Mock-infected cells were used as controls.

When compared to mock-infected cells, HUVEC infected with ANDV, HTNV, and PHV were observed to upregulate levels of CCL5 RNA at 12, 24, and 72 h PI (**Figure 5A**). Additionally, transcription of IL-6 was significantly increased in HTNV and PHV-infected HUVEC at 24 h PI and increased for all viruses at 72 h PI, when compared to mock-infected cells ($p < 0.01$; **Figure 5B**). Also, the levels of CCL5 and IL-6 were significantly increased in culture supernatant of HUVEC infected with ANDV,

HTNV, and PHV at 72 h PI when compared to mock-infected cells ($p < 0.01$; **Figures 5C,D**, respectively).

During the course of these experiments, we observed that the maximum concentration of CCL5 and IL-6 found in supernatants of hantavirus-infected HUVEC cultures was 12 and 50 ng/mL, respectively. Therefore, we used these concentrations to treat uninfected HUVEC in order to assess the effect of CCL5 or IL-6 on THBS1 accumulation in the extracellular matrix. No changes in THBS1 accumulation were observed upon treatment of HUVEC with CCL5 or IL-6 when compared to untreated controls, as determined by Western blot analysis (**Figure 5E**), suggesting that the effect was not strictly the result of these inflammatory cytokines.

DISCUSSION

Although it has been suggested that the high viral loads observed in the endothelial cells of those with HFRS and HPS is related to disease pathogenesis (Yi et al., 2014; Bellomo et al., 2015), the mechanisms of pathogenesis remains unknown. Hantaviruses are not cytopathic *in vitro* (Pensiero et al., 1992; Sundstrom et al., 2001; Khaiboullina et al., 2004), and no pathological evidence of virus-induced cell death has been reported in tissues from fatal hantavirus cases (Zhang et al., 1987; Zaki et al., 1995). The absence of virus-induced cytopathicity *in vitro* is in stark contrast with the severe vascular disorders observed during HPS and HFRS (Sargianou et al., 2012; Manigold and Vial, 2014). For this reason, it has been suggested that hantavirus pathology more likely involves a dysregulation of the infected endothelial cell's ability to support their primary function as a blood-tissue barrier (Connolly-Andersen et al., 2014).

In this report, we have demonstrated the *in vitro* suppression of THBS1 transcription in endothelial cells through hantavirus infection. We additionally show that hantavirus infection reduces THBS1 protein accumulation in the extracellular matrix of endothelial cells. Importantly, the inhibitory effects of hantavirus infection were strain specific, whereby, the pathogenic hantaviruses ANDV and HTNV were more effective in suppressing the extracellular matrix accumulation of THBS1. On the other hand, the effects of the non-pathogenic PHV were less pronounced. Additionally, suppression of THBS1 was dependent on virus replication and did not require the presence of CCL5 or IL-6. Our data suggests that hantavirus infection directly influences the expression and accumulation of THBS1, an endothelial cell protein with putative roles in cell adhesion, platelet aggregation, and the regulation of fibrinolysis (Silverstein et al., 1984; Roberts et al., 2010). These data support a mechanism of disease pathogenesis characterized by decreased expression

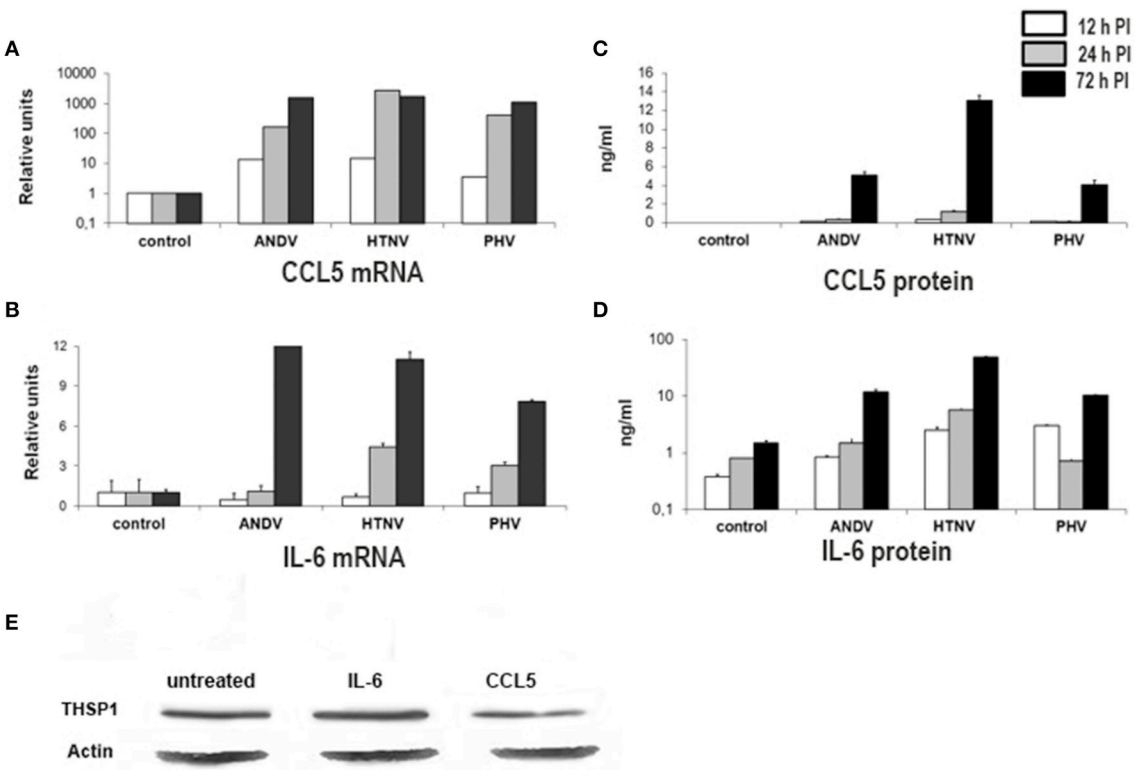

FIGURE 5 | Transcriptional activity and secretion of CCL5 and IL-6 in hantavirus-infected cells. Total RNA and cell culture supernatants were collected from hantavirus infected cells at 12, 24, and 72 h PI. Transcription of CCL5 **(A)** and IL-6 **(B)** was analyzed using TaqMan. Levels of CCL5 **(C)** and IL-6 **(D)** in cell culture media were determined using ELISA. Effect of IL-6 and CCL5 on THBS1 expression in HUVEC by Western blot **(E)**. Total proteins were collected from cells either untreated, treated with 50 ng/mL IL-6 or 12 ng/mL of CCL5 for 72 h. Proteins were separated by SDS-PAGE, transferred onto PVDF membrane and probed with anti-THBS1 mAb antibodies followed by anti-mouse-HRP conjugated secondary antibody.

of THBS1 in infected endothelial cells, which in turn leads to a perturbation of normal vascular integrity, and possibly hemostasis, which ultimately contribute to the severe vascular disorders characteristic of pathogenic hantavirus pathology (Adams, 1997; Sargianou et al., 2012; Manigold and Vial, 2014). This pathology may include loss of renal function, capillary leakage, and thrombocytopenia (Thakar et al., 2005; Garg et al., 2011; Sargianou et al., 2012; Vaheri et al., 2013; Latus et al., 2015).

Among the many functions of THBS1, three are of particular interest in relation to hantavirus pathogenesis: first, THBS1 controls vW factor multimer sizes (Xie et al., 2001; Pimanda et al., 2004). vW factor mediates adhesion of platelets at sites of vascular injury; however, only the very large vW factor multimers are effective in promoting platelet adhesion (Sadler, 1998; Xie et al., 2001). THBS1 can reduce intersubunit disulfide bonds of vW factor leading to formation of smaller multimers that have significantly lower activity for platelet aggregation, thus preventing thrombosis (Thakar et al., 2005) and protecting the integrity of vascular endothelium (Xie et al., 2001).

Second, THBS1 suppresses plasminogen activator inhibitor, which allows tissue plasminogen activator and urokinase plasminogen activator to convert plasminogen into plasmin, the principal enzyme responsible for fibrin degradation in the process of fibrinolysis (Silverstein et al., 1986; Hogg et al., 1992; Rabhi-Sabile et al., 1998). Accordingly, an absence of THBS1 and a decrease in fibrinolysis, may lead to excessive coagulation and consumption of clotting factors (Lee, 1987). However, THBS1 is also a slow tight-binding inhibitor of plasmin (Hogg et al., 1992). Therefore, in one context THBS1 may increase platelet aggregation, but in another context prevents blood clots from developing and becoming problematic. In fact, THBS1 released from activated platelets participates both in the formation as well as the resolution of the fibrin clot (Adams, 1997). Accordingly, THBS1 likely influences the balance between procoagulation and anticoagulation proteins, in a context-dependent manner, thus regulating blood flow.

Finally, Bauer et al. reported that circulating THBS1 blocks endothelial-dependent decreases in blood pressure by limiting the production of the diffusible vasodilator nitric oxide (NO; Bauer et al., 2010). Consistent with this observation, and in the context of this study, Liu et al., reported decreased circulating levels of THBS1 in subjects with HFRS (Liu et al., 2008). These data offer an alternative mechanism that is not directly related to the coagulation cascade. By suppressing THBS1 expression in endothelial cells, hantaviruses may affect normal blood flow by decreasing thrombolysis, promoting platelet aggregation, and increasing vascular permeability. However, it should be noted that blood homeostasis is a dynamic process, involving many proteins. THBS1 is known to interact with as many as 50 different proteins (Resovi et al., 2014), and therefore, the precise mechanism of THBS1's involvement may be indirect or through undetermined interactions.

In a previous study to characterized ANDV infection in its native host, Peromyscus maniculatus, Spengler et al., reported that the largely anti-inflammatory cytokine TGFβ was markedly upregulated (Spengler et al., 2013). They further speculate that this observation may explain why ANDV infection in not associated with any clinical signs of pathology in its native host. We observed a paucity of THBS1 accumulation in the extracellular matrix of ANDV-infected HUVEC, however, when ANDV-infected cells were treated with TGFβ, a significant increase in the accumulation of THBS1 was observed (**Figure 1F**). We also observed this effect to be suppressed in the presence of the proinflammatory cytokine TNFα. Indeed, it has been reported that TNFα antagonizes the activities of TGFβ and is believed to play an essential role in maintaining the stability of extracellular matrix proteins (Verrecchia et al., 2000; Verrecchia and Mauviel, 2004).

As shown in **Figure 2**, treatment of HUVEC with TNFα also suppressed THBS1 gene transcription in uninfected endothelial cells. In fact, THBS1 distribution in the extracellular matrix of TNFα-treated and uninfected endothelial cells appear similar to that of untreated endothelial cells infected with pathogenic hantaviruses (**Figure 1**). Several signal transduction pathways are activated by TNFα that can affect THBS1 accumulation, including c-Jun and NF-κB transcription activation factors (Manna et al., 1998; Rahman, 2000). For instance, Kim and Hong reported that decreased THBS1, in response to the inflammatory stimuli phorbol 12-myristate 13-acetate, was regulated by c-Jun (Kim and Hong, 2000). Our data suggest that hantavirus infection activates c-Jun; therefore, it is possible that TNFα may synergizes hantavirus-triggered activation of transcription factors including those involved in THBS1 accumulation. Although TNFα is not directly produced by endothelial cells, an increase in serum TNFα has been reported in the blood of hantavirus infected cases (Krakauer et al., 1995).

While non-pathogenic PHV suppressed the accumulation of THBS1 protein in endothelial cells, the effect was less pronounced when compared to pathogenic strains. Similarly, PHV was not a strong suppressor of THBS1 gene transcription when compared to pathogenic hantaviruses. These data suggest that the non-pathogenic hantavirus have less effect on the expression of endothelial cell proteins involved in the control of blood homeostasis than pathogenic hantaviruses. These observations potentially identify a mechanism for the different pathologies of different hantavirus strains.

Finally, our data suggest that hantavirus replication is required for the suppression of THBS1 accumulation. Although CCL5 and IL-6 upregulation did not suppress THBS1 accumulation, it's possible that c-Jun may be involved. It has been shown for several other viruses that viral proteins can modulate cell protein transcription by interacting with transcription activation factors. For example, the major early regulatory protein large T-Ag, of John Cunningham virus (JC virus), can interact with AP-1 transcription activation proteins (Kim et al., 2003). Also, hepatitis C virus has been shown to activate c-Jun N-terminal kinase and p38 mitogen-activated protein (MAP) kinase (Erhardt et al., 2002). The mechanisms of transcription factor activation by hantaviruses remain to be determined; however, it is possible that viral proteins and/or RNA-protein complexes may be involved as suggested by the activation of c-Jun.

In summary, our data suggest that pathogenic Andes and Hantaan virus infection of endothelial cells suppress the

accumulation of THBS1 in the extracellular matrix while the non-pathogenic Prospect Hill strain display little inhibition. THBS1 interacts with numerous members of the coagulation cascade, suggesting a mechanism of pathology. However, the pathophysiology of hantavirus infection is complex and involves not only the dysregulation of coagulation, hyperinflammatory immune responses likely play an important role, that may be independent of coagulation. Although the involvement of THBS1 suggests a potential disease mechanism, further studies will be required to fully understand its involvement in the disease process.

AUTHOR CONTRIBUTIONS

SK and SS conceived of the project, SK AR, and SM conducted experiments, SK and VL wrote the manuscript.

ACKNOWLEDGMENTS

This study was supported in part by Russian Science Foundation grant 15-14-00016. The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University and subsidy allocated to Kazan Federal University for the state assignment in the sphere of scientific activities. Some of the experiments were conducted using the equipment of the Interdisciplinary Center for Collective Use of Kazan Federal University, supported by the Ministry of Education of Russia (ID RFMEFI59414X0003), Interdisciplinary Center for Analytical Microscopy and Pharmaceutical Research and Education Center, Kazan (Volga Region) Federal University, Kazan, Russia. An award from the Department of Defense (DOD) supports work conducted in the laboratory of VL (grant PR131133). We thank Dr. Mark A. Hall for helpful discussions and Dr. David A. Schooley for his critical reading of the manuscript.

REFERENCES

Adams, J. C. (1997). Thrombospondin-1. *Int. J. Biochem. Cell Biol.* 29, 861–865.

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., et al. (1990). A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.* 9, 1897–1906.

Albo, D., Berger, D. H., Wang, T. N., Hu, X., Rothman, V., and Tuszynski, G. P. (1997). Thrombospondin-1 and transforming growth factor-beta l promote breast tumor cell invasion through up-regulation of the plasminogen/plasmin system. *Surgery* 122, 493–499. discussion: 499–500.

Bauer, E. M., Qin, Y., Miller, T. W., Bandle, R. W., Csanyi, G., and Isenberg, J. S. (2010). Thrombospondin-1 supports blood pressure by limiting eNOS activation and endothelial-dependent vasorelaxation. *Cardiovasc. Res.* 88, 471–481. doi: 10.1093/cvr/cvq218

Bein, K., and Simons, M. (2000). Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. *J. Biol. Chem.* 275, 32167–32173. doi: 10.1074/jbc.M003834200

Bellomo, C. M., Pires-Marczeski, F. C., and Padula, P. J. (2015). Viral load of patients with hantavirus pulmonary syndrome in Argentina. *J. Med. Virol.* 87, 1823–1830. doi: 10.1002/jmv.24260

Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K., and Tjian, R. (1987). Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* 238, 1386–1392.

Bondu, V., Schrader, R., Gawinowicz, M. A., McGuire, P., Lawrence, D. A., Hjelle, B., et al. (2015). Elevated cytokines, thrombin and PAI-1 in severe HCPS patients due to Sin Nombre virus. *Viruses* 7, 559–589. doi: 10.3390/v7020559

Chu, L. Y., Ramakrishnan, D. P., and Silverstein, R. L. (2013). Thrombospondin-1 modulates VEGF signaling via CD36 by recruiting SHP-1 to VEGFR2 complex in microvascular endothelial cells. *Blood* 122, 1822–1832. doi: 10.1182/blood-2013-01-482315

Cinatl, J. Jr., Bittoova, M., Margraf, S., Vogel, J. U., Cinatl, J., Preiser, W., et al. (2000). Cytomegalovirus infection decreases expression of thrombospondin-1 and -2 in cultured human retinal glial cells: effects of antiviral agents. *J. Infect. Dis.* 182, 643–651. doi: 10.1086/315779

Cirino, G., Napoli, C., Bucci, M., and Cicala, C. (2000). Inflammation-coagulation network: are serine protease receptors the knot? *Trends Pharmacol. Sci.* 21, 170–172. doi: 10.1016/S0165-6147(00)01469-3

Connolly-Andersen, A. M., Sundberg, E., Ahlm, C., Hultdin, J., Baudin, M., Larsson, J., et al. (2015). Increased thrombopoiesis and platelet activation in hantavirus-infected patients. *J. Infect. Dis.* 212, 1061–1069. doi: 10.1093/infdis/jiv161

Connolly-Andersen, A. M., Thunberg, T., and Ahlm, C. (2014). Endothelial activation and repair during hantavirus infection: association with disease outcome. *Open Forum Infect. Dis.* 1:ofu027. doi: 10.1093/ofid/ofu027

Curran, T., and Franza, B. R. Jr. (1988). Fos and Jun the AP-1 connection. *Cell* 55, 395–397.

Davidovich, I. M., Parshina, T. A., and Utkin, V. N. (1993). [Von Willebrand factor, antithrombin III and 5′-nucleotidase in the blood and the athrombogenic properties of the vascular wall in patients with hemorrhagic fever with renal syndrome]. *Ter. Arkh.* 65, 22–25.

Dugina, T. N., Kiseleva, E. V., Chistov, I. V., Umarova, B. A., and Strukova, S. M. (2002). Receptors of the PAR family as a link between blood coagulation and inflammation. *Biochemistry* 67, 65–74. doi: 10.1023/A:1013952114485

Enria, D. A., Briggiler, A. M., Pini, N., and Levis, S. (2001). Clinical manifestations of New World hantaviruses. *Curr. Top. Microbiol. Immunol.* 256, 117–134. doi: 10.1007/978-3-642-56753-7_7

Erhardt, A., Hassan, M., Heintges, T., and Häussinger, D. (2002). Hepatitis C virus core protein induces cell proliferation and activates ERK, JNK, and p38 MAP kinases together with the MAP kinase phosphatase MKP-1 in a HepG2 Tet-Off cell line. *Virology* 292, 272–284. doi: 10.1006/viro.2001.1227

Garg, P., Yang, S., Liu, A., Pallero, M. A., Buchsbaum, D. J., Mosher, D. F., et al. (2011). Thrombospondin-1 opens the paracellular pathway in pulmonary microvascular endothelia through EGFR/ErbB2 activation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 301, L79–L90. doi: 10.1152/ajplung.00287.2010

Geimonen, E., Neff, S., Raymond, T., Kocer, S. S., Gavrilovskaya, I. N., and Mackow, E. R. (2002). Pathogenic and nonpathogenic hantaviruses differentially regulate endothelial cell responses. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13837–13842. doi: 10.1073/pnas.192298899

Goicoechea, S., Orr, A. W., Pallero, M. A., Eggleton, P., and Murphy-Ullrich, J. E. (2000). Thrombospondin mediates focal adhesion disassembly through interactions with cell surface calreticulin. *J. Biol. Chem.* 275, 36358–36368. doi: 10.1074/jbc.M005951200

Greenwood, J. A., Pallero, M. A., Theibert, A. B., and Murphy-Ullrich, J. E. (1998). Thrombospondin signaling of focal adhesion disassembly requires activation of phosphoinositide 3-kinase. *J. Biol. Chem.* 273, 1755–1763.

Hogg, P. J., Owensby, D. A., and Chesterman, C. N. (1993). Thrombospondin 1 is a tight-binding competitive inhibitor of neutrophil cathepsin G. Determination of the kinetic mechanism of inhibition and localization of cathepsin G binding to the thrombospondin 1 type 3 repeats. *J. Biol. Chem.* 268, 21811–21818.

Hogg, P. J., Stenflo, J., and Mosher, D. F. (1992). Thrombospondin is a slow tight-binding inhibitor of plasmin. *Biochemistry* 31, 265–269.

Jenison, S., Hjelle, B., Simpson, S., Hallin, G., Feddersen, R., and Koster, F. (1995). Hantavirus pulmonary syndrome: clinical, diagnostic, and virologic aspects. *Semin. Respir. Infect.* 10, 259–269.

Kanerva, M., Mustonen, J., and Vaheri, A. (1998). Pathogenesis of puumala and other hantavirus infections. *Rev. Med. Virol.* 8, 67–86.

Khaiboullina, S. F., Rizvanov, A. A., Otteson, E., Miyazato, A., Maciejewski, J., and St Jeor, S. (2004). Regulation of cellular gene expression in endothelial cells by sin nombre and prospect hill viruses. *Viral Immunol.* 17, 234–251. doi: 10.1089/0882824041310504

Khan, A. S., Khabbaz, R. F., Armstrong, L. R., Holman, R. C., Bauer, S. P., Graber, J., et al. (1996). Hantavirus pulmonary syndrome: the first 100 US cases. *J. Infect. Dis.* 173, 1297–1303.

Kim, J., Woolridge, S., Biffi, R., Borghi, E., Lassak, A., Ferrante, P., et al. (2003). Members of the AP-1 family, c-Jun and c-Fos, functionally interact with JC virus early regulatory protein large T antigen. *J. Virol.* 77, 5241–5252. doi: 10.1128/JVI.77.9.5241-5252.2003

Kim, S. A., and Hong, K. J. (2000). Responsive site on the thrombospondin-1 promotor to down-regulation by phorbol 12-myristate 13-acetate in porcine aortic endothelial cells. *Exp. Mol. Med.* 32, 135–140. doi: 10.1038/emm.2000.23

Kinoshita, S., Akira, S., and Kishimoto, T. (1992). A member of the C/EBP family, NF-IL6 beta, forms a heterodimer and transcriptionally synergizes with NF-IL6. *Proc. Natl. Acad. Sci. U.S.A.* 89, 1473–1476.

Krakauer, T., Leduc, J. W., and Krakauer, H. (1995). Serum levels of tumor necrosis factor-alpha, interleukin-1, and interleukin-6 in hemorrhagic fever with renal syndrome. *Viral Immunol.* 8, 75–79.

Laine, O. K., Koskela, S. M., Outinen, T. K., Joutsi-Korhonen, L., Huhtala, H., Vaheri, A., et al. (2014). Plasma pentraxin-3 and coagulation and fibrinolysis variables during acute Puumala hantavirus infection and associated thrombocytopenia. *Blood Coagul. Fibrinolysis* 25, 612–617. doi: 10.1097/MBC.0000000000000117

Latus, J., Kitterer, D., Segerer, S., Artunc, F., Alscher, M. D., and Braun, N. (2015). Severe thrombocytopenia in hantavirus-induced nephropathia epidemica. *Infection* 43, 83–87. doi: 10.1007/s15010-014-0699-9

Lawler, J., and Detmar, M. (2004). Tumor progression the effects of thrombospondin-1 and -2. *Int. J. Biochem. Cell Biol.* 36, 1038–1045. doi: 10.1016/j.biocel.2004.01.008

Lee, M. (1987). Coagulopathy in patients with hemorrhagic fever with renal syndrome. *J. Korean Med. Sci.* 2, 201–211. doi: 10.3346/jkms.1987.2.4.201

Lee, M., Kim, B. K., Kim, S., Park, S., Han, J. S., Kim, S. T., et al. (1989). Coagulopathy in hemorrhagic fever with renal syndrome (Korean hemorrhagic fever). *Rev. Infect. Dis.* 11(Suppl. 4), S877–S883.

Linderholm, M., and Elgh, F. (2001). Clinical characteristics of hantavirus infections on the Eurasian continent. *Curr. Top. Microbiol. Immunol.* 256, 135–151. doi: 10.1007/978-3-642-56753-7_8

Liu, A., Mosher, D. F., Murphy-Ullrich, J. E., and Goldblum, S. E. (2009). The counteradhesive proteins, thrombospondin 1 and SPARC/osteonectin, open the tyrosine phosphorylation-responsive paracellular pathway in pulmonary vascular endothelia. *Microvasc. Res.* 77, 13–20. doi: 10.1016/j.mvr.2008.08.008

Liu, Z., Zhao, Q., Han, Q., Gao, M., and Zhang, N. (2008). Serum thrombospondin-1 is altered in patients with hemorrhagic fever with renal syndrome. *J. Med. Virol.* 80, 1799–1803. doi: 10.1002/jmv.21270

Lopez-Dee, Z., Pidcock, K., and Gutierrez, L. S. (2011). Thrombospondin-1: multiple paths to inflammation. *Mediators Inflamm.* 2011:296069. doi: 10.1155/2011/296069

Manigold, T., and Vial, P. (2014). Human hantavirus infections: epidemiology, clinical features, pathogenesis and immunology. *Swiss Med. Wkly.* 144:w13937. doi: 10.4414/smw.2014.13937

Manna, S. K., Zhang, H. J., Yan, T., Oberley, L. W., and Aggarwal, B. B. (1998). Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J. Biol. Chem.* 273, 13245–13254.

Mast, A. E., Stadanlick, J. E., Lockett, J. M., Dietzen, D. J., Hasty, K. A., and Hall, C. L. (2000). Tissue factor pathway inhibitor binds to platelet thrombospondin-1. *J. Biol. Chem.* 275, 31715–31721. doi: 10.1074/jbc.M006595200

McPherson, J., Sage, H., and Bornstein, P. (1981). Isolation and characterization of a glycoprotein secreted by aortic endothelial cells in culture. Apparent identity with platelet thrombospondin. *J. Biol. Chem.* 256, 11330–11336.

Morandi, V., Cherradi, S. E., Lambert, S., Fauvel-Laféve, F., Legrand, Y. J., and Legrand, C. (1994). Proinflammatory cytokines (interleukin-1 beta and tumor necrosis factor-alpha) down regulate synthesis and secretion of thrombospondin by human endothelial cells. *J. Cell. Physiol.* 160, 367–377. doi: 10.1002/jcp.1041600218

Nakagawa, T., Lan, H. Y., Glushakova, O., Zhu, H. J., Kang, D. H., Schreiner, G. F., et al. (2005). Role of ERK1/2 and p38 mitogen-activated protein kinases in the regulation of thrombospondin-1 by TGF-beta1 in rat proximal tubular cells and mouse fibroblasts. *J. Am. Soc. Nephrol.* 16, 899–904. doi: 10.1681/ASN.2004080689

Negoescu, A., Lafeuillade, B., Pellerin, S., Chambaz, E. M., and Feige, J. J. (1995). Transforming growth factors beta stimulate both thrombospondin-1 and CISP/thrombospondin-2 synthesis by bovine adrenocortical cells. *Exp. Cell Res.* 217, 404–409. doi: 10.1006/excr.1995.1103

Pensiero, M. N., Sharefkin, J. B., Dieffenbach, C. W., and Hay, J. (1992). Hantaan virus infection of human endothelial cells. *J. Virol.* 66, 5929–5936.

Peters, C. J., and Khan, A. S. (2002). Hantavirus pulmonary syndrome: the new American hemorrhagic fever. *Clin. Infect. Dis.* 34, 1224–1231. doi: 10.1086/339864

Peters, C. J., Simpson, G. L., and Levy, H. (1999). Spectrum of hantavirus infection: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. *Annu. Rev. Med.* 50, 531–545. doi: 10.1146/annurev.med.50.1.531

Pimanda, J. E., Ganderton, T., Maekawa, A., Yap, C. L., Lawler, J., Kershaw, G., et al. (2004). Role of thrombospondin-1 in control of von Willebrand factor multimer size in mice. *J. Biol. Chem.* 279, 21439–21448. doi: 10.1074/jbc.M313560200

Prakash, P., Kulkarni, P. P., and Chauhan, A. K. (2015). Thrombospondin 1 requires von Willebrand factor to modulate arterial thrombosis in mice. *Blood* 125, 399–406. doi: 10.1182/blood-2014-06-581942

Rabhi-Sabile, S., de Romeuf, C., and Pidard, D. (1998). On the mechanism of plasmin-induced aggregation of human platelets: implication of secreted von Willebrand factor. *Thromb. Haemost.* 79, 1191–1198.

Rahman, I. (2000). Regulation of nuclear factor-kappa B, activator protein-1, and glutathione levels by tumor necrosis factor-alpha and dexamethasone in alveolar epithelial cells. *Biochem. Pharmacol.* 60, 1041–1049. doi: 10.1016/S0006-2952(00)00392-0

Rasche, F. M., Uhel, B., Krüger, D. H., Karges, W., Czock, D., Hampl, W., et al. (2004). Thrombocytopenia and acute renal failure in Puumala hantavirus infections. *Emerging Infect. Dis.* 10, 1420–1425. doi: 10.3201/eid1008.031069

Resovi, A., Pinessi, D., Chiorino, G., and Taraboletti, G. (2014). Current understanding of the thrombospondin-1 interactome. *Matrix Biol.* 37, 83–91. doi: 10.1016/j.matbio.2014.01.012

Roberts, W., Magwenzi, S., Aburima, A., and Naseem, K. M. (2010). Thrombospondin-1 induces platelet activation through CD36-dependent inhibition of the cAMP/protein kinase A signaling cascade. *Blood* 116, 4297–4306. doi: 10.1182/blood-2010-01-265561

Sadeghi, M., Eckerle, I., Daniel, V., Burkhardt, U., Opelz, G., and Schnitzler, P. (2011). Cytokine expression during early and late phase of acute Puumala hantavirus infection. *BMC Immunol.* 12:65. doi: 10.1186/1471-2172-12-65

Sadler, J. E. (1998). Biochemistry and genetics of von Willebrand factor. *Annu. Rev. Biochem.* 67, 395–424. doi: 10.1146/annurev.biochem.67.1.395

Sargianou, M., Watson, D. C., Chra, P., Papa, A., Starakis, I., Gogos, C., et al. (2012). Hantavirus infections for the clinician: from case presentation to diagnosis and treatment. *Crit. Rev. Microbiol.* 38, 317–329. doi: 10.3109/1040841X.2012.673553

Saumet, A., de Jesus, N., Legrand, C., and Dubernard, V. (2002). Association of thrombospondin-1 with the actin cytoskeleton of human thrombin-activated platelets through an alphaIIbbeta3- or CD36-independent mechanism. *Biochem. J.* 363(Pt. 3), 473–482. doi: 10.1042/bj3630473

Schmaljohn, C. (2007). "Biodefense challenges," in *The Biomedical and Life Sciences Collection* (London: Henry Stewart Talks Ltd). Available online at: https://hstalks.com/bs/361/

Schmaljohn, C. S., and Nichol, S. T. (2001). *Hantaviruses*. Berlin; New York, NY: Springer.

Schultz-Cherry, S., Chen, H., Mosher, D. F., Misenheimer, T. M., Krutzsch, H. C., Roberts, D. D., et al. (1995). Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. *J. Biol. Chem.* 270, 7304–7310.

Silverstein, R. L., Harpel, P. C., and Nachman, R. L. (1986). Tissue plasminogen activator and urokinase enhance the binding of plasminogen to thrombospondin. *J. Biol. Chem.* 261, 9959–9965.

Silverstein, R. L., Leung, L. L., Harpel, P. C., and Nachman, R. L. (1984). Complex formation of platelet thrombospondin with plasminogen. Modulation of activation by tissue activator. *J. Clin. Invest.* 74, 1625–1633. doi: 10.1172/JCI111578

Slofstra, S. H., Spek, C. A., and ten Cate, H. (2003). Disseminated intravascular coagulation. *Hematol. J.* 4, 295–302. doi: 10.1038/sj.thj.6200263

Spengler, J. R., Haddock, E., Gardner, D., Hjelle, B., Feldmann, H., and Prescott, J. (2013). Experimental Andes virus infection in deer mice: characteristics of infection and clearance in a heterologous rodent host. *PLoS ONE* 8:e55310. doi: 10.1371/journal.pone.0055310

Strandin, T., Hepojoki, J., Laine, O., Mäkelä, S., Klingström, J., Lundkvist, Å., et al. (2016). Interferons induce STAT1-dependent expression of tissue plasminogen activator, a pathogenicity factor in Puumala hantavirus disease. *J. Infect. Dis.* 213, 1632–1641. doi: 10.1093/infdis/jiv764

Sundberg, E., Hultdin, J., Nilsson, S., and Ahlm, C. (2011). Evidence of disseminated intravascular coagulation in a hemorrhagic fever with renal syndrome-scoring models and severe illness. *PLoS ONE* 6:e21134. doi: 10.1371/journal.pone.0021134

Sundstrom, J. B., McMullan, L. K., Spiropoulou, C. F., Hooper, W. C., Ansari, A. A., Peters, C. J., et al. (2001). Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells. *J. Virol.* 75, 6070–6085. doi: 10.1128/JVI.75.13.6070-6085.2001

Thakar, C. V., Zahedi, K., Revelo, M. P., Wang, Z., Burnham, C. E., Barone, S., et al. (2005). Identification of thrombospondin 1 (TSP-1) as a novel mediator of cell injury in kidney ischemia. *J. Clin. Invest.* 115, 3451–3459. doi: 10.1172/JCI25461

Vaheri, A., Strandin, T., Hepojoki, J., Sironen, T., Henttonen, H., Mäkelä, S., et al. (2013). Uncovering the mysteries of hantavirus infections. *Nat. Rev. Microbiol.* 11, 539–550. doi: 10.1038/nrmicro3066

Verrecchia, F., and Mauviel, A. (2004). TGF-beta and TNF-alpha: antagonistic cytokines controlling type I collagen gene expression. *Cell. Signal.* 16, 873–880. doi: 10.1016/j.cellsig.2004.02.007

Verrecchia, F., Pessah, M., Atfi, A., and Mauviel, A. (2000). Tumor necrosis factor-alpha inhibits transforming growth factor-beta /Smad signaling in human dermal fibroblasts via AP-1 activation. *J. Biol. Chem.* 275, 30226–30231. doi: 10.1074/jbc.M005310200

Xie, L., Chesterman, C. N., and Hogg, P. J. (2001). Control of von Willebrand factor multimer size by thrombospondin-1. *J. Exp. Med.* 193, 1341–1349. doi: 10.1084/jem.193.12.1341

Yang, A. L., Zhou, H. J., Lin, Y., Luo, J. K., Cui, H. J., Tang, T., et al. (2012). Thrombin promotes the expression of thrombospondin-1 and -2 in a rat model of intracerebral hemorrhage. *J. Neurol. Sci.* 323, 141–146. doi: 10.1016/j.jns.2012.09.002

Yi, J., Zhang, Y., Zhang, Y., Ma, Y., Zhang, C., Li, Q., et al. (2014). Increased plasma cell-free DNA level during HTNV infection: correlation with disease severity and virus load. *Viruses* 6, 2723–2734. doi: 10.3390/v6072723

Zaki, S. R., Greer, P. W., Coffield, L. M., Goldsmith, C. S., Nolte, K. B., Foucar, K., et al. (1995). Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. *Am. J. Pathol.* 146, 552–579.

Zhang, X. L., Wang, X. H., and Liu, C. Z. (1987). [Detection of viral antigens in various organs in 14 fatal cases of epidemic hemorrhagic fever]. *Zhonghua Nei Ke Za Zhi* 26, 461–463, 509–510.

Zhao, Y., Olonisakin, T. F., Xiong, Z., Hulver, M., Sayeed, S., Yu, M. T., et al. (2015). Thrombospondin-1 restrains neutrophil granule serine protease function and regulates the innate immune response during Klebsiella pneumoniae infection. *Mucosal Immunol.* 8, 896–905. doi: 10.1038/mi.2014.120

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 31 August 2015
doi: 10.3389/fimmu.2015.00432

Multiplex analysis of serum cytokines in humans with hantavirus pulmonary syndrome

Sergey P. Morzunov[1], Svetlana F. Khaiboullina[2,3], Stephen St. Jeor[4], Albert A. Rizvanov[2] and Vincent C. Lombardi[3,5]**

[1] Department of Pathology, School of Medicine, University of Nevada, Reno, NV, USA, [2] Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia, [3] Whittemore Peterson Institute, Reno, NV, USA, [4] Department of Microbiology and Immunology, University of Nevada, Reno, NV, USA, [5] Department of Biochemistry, School of Medicine, University of Nevada, Reno, NV, USA

OPEN ACCESS

Edited by:
Gayane Manukyan,
National Academy of Sciences of the Republic of Armenia, Armenia

Reviewed by:
Joshua J. Obar,
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Rudra Bhowmick,
Oklahoma State University, USA
Levon Abrahamyan,
Université de Montréal, Canada

***Correspondence:**
Albert A. Rizvanov,
Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, 18 Kremlevskaya Street, Kazan 420008, Tatarstan, Russia
albert.rizvanov@kpfu.ru;
Vincent C. Lombardi,
Department of Biochemistry and Molecular Biology, University of Nevada, 1664 N Virginia Street, Reno, NV MS 0330, USA
vlombardi@medicine.nevada.edu

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: 03 June 2015
Accepted: 09 August 2015
Published: 31 August 2015

Citation:
Morzunov SP, Khaiboullina SF, St. Jeor S, Rizvanov AA and Lombardi VC (2015) Multiplex analysis of serum cytokines in humans with hantavirus pulmonary syndrome. Front. Immunol. 6:432. doi: 10.3389/fimmu.2015.00432

Hantavirus pulmonary syndrome (HPS) is an acute zoonotic disease transmitted primarily through inhalation of virus-contaminated aerosols. Hantavirus infection of endothelial cells leads to increased vascular permeability without a visible cytopathic effect. For this reason, it has been suggested that the pathogenesis of HPS is indirect with immune responses, such as cytokine production, playing a dominant role. In order to investigate their potential contribution to HPS pathogenesis, we analyzed the serum of hantavirus-infected subjects and healthy controls for 68 different cytokines, chemokines, angiogenic, and growth factors. Our analysis identified differential expression of cytokines that promote tissue migration of mononuclear cells including T lymphocytes, natural killer cells, and dendritic cells. Additionally, we observed a significant upregulation of cytokines known to regulate leukocyte migration and subsequent repair of lung tissue, as well as cytokines known to increase endothelial monolayer permeability and facilitate leukocyte transendothelial migration. Conversely, we observed a downregulation of cytokines associated with platelet numbers and function, consistent with the thrombocytopenia observed in subjects with HPS. This study corroborates clinical findings and extends our current knowledge regarding immunological and laboratory findings in subjects with HPS.

Keywords: hantavirus pulmonary syndrome, serum, cytokines, chemokines, growth factors, immune response, hantaviruses

Introduction

Hantavirus pulmonary syndrome (HPS) is a severe life threatening disease caused by members of the genus *Hantavirus*. In the United States, these members include Sin Nombre virus, Bayou virus, Black Creek Canal virus, and New York virus, while South American members include Andes virus and Laguna Negra virus (1–5). Although HPS was first diagnosed as a clinical entity in 1993 in response to the four corners outbreak (6), retrospective studies have identified hantavirus-associated fatalities as early as 1978 (7). HPS cases have been reported in 34 states with the majority occurring in the Southwestern states; however, several have been reported in the Northwestern and Midwestern states. Through April 2014, the Center for Disease Control and Prevention has confirmed 639 total cases of HPS in the U.S., with the majority occurring in New Mexico (94 cases), Colorado (81 cases), and Arizona (72 cases) (8). Although the prevalence of HPS is low in the U.S., 36% of all reported HPS cases have resulted in death, underscoring the potential impact to public health.

Clinically, HPS manifests with fatigue, fever, muscle pain, headache, dizziness, nausea, and vomiting (9). Soon after onset, individuals present with bilateral diffuse interstitial edema resembling acute respiratory distress syndrome (10). Rapidly progressing pulmonary edema, myocardial depression, and hypovolemia are the leading cause of death (11). There is no specific treatment for HPS; therefore, medical care is mainly supportive with early diagnosis resulting in more successful outcomes.

Hantaviruses do not produce a visible cytopathic effect; consequently, it is believed that cytokines produced by infected cells either directly or indirectly lead to a compromised endothelial monolayer, which in turn, leads to vascular leakage. Indeed, increased numbers of cytokine-producing cells have been observed in lung and spleen tissue of HPS cases (12). We as well as others have demonstrated that endothelial cells produce the chemokines, CCL5 and CXCL10, when infected with Sin Nombre virus (13, 14). These cytokines are strong chemoattractants for mononuclear leukocytes including monocytes, lymphocytes, and natural killer (NK) cells (15, 16). Expression of these chemokines may explain the postmortem observation of monocytic interstitial pneumonia in fatal HPS cases; however, it remains to be determined whether these chemokines are expressed during active HPS. In contrast to CCL5 and CXCL10 and atypical of most viral infections, *in vitro* culture studies show that only a slight upregulation of type I interferon (IFN) is observed when endothelial cells are infected with hantaviruses. These data are also consistent with clinical observations that suggest that a robust IFN-α response is not characteristic of hantavirus infection (17, 18).

Although limited data exist regarding cytokine expression in subjects with HPS, a study by Borges et al. evaluated the concentrations of 11 serum analytes by ELISA. A cytokine profile was reported that defined the differential expression of a selected number of Th1 and Th2 cytokines (19). Specifically, they observed significantly elevated levels of IL-6, IFN-γ, sIL-2R, TNF-α, and decreased IL-10 when compared to controls, suggesting that activation of Th1 and Th2-type immune responses are involved. While ELISA is commonly used for such studies, it has limitations such as the necessity of a large sample volume and this issue is compounded when one wishes to analyze multiple analytes. High-throughput multiplex analysis by Luminex xMAP technology allows the simultaneous detection and quantitation of many analytes and uses a small amount of serum or plasma. In the present study, we utilized Luminex xMAP technology to conduct a comprehensive evaluation of 68 different cytokines, chemokines, angiogenic, and growth factors (hereafter referred to collectively as cytokines) in subjects with HPS, including 38 cytokines previously not investigated in association with this disease. Changes in 40 cytokines were detected in the serum of subjects with HPS when compared to healthy controls; 25 cytokines were significantly upregulated while15 were downregulated. A subset of these cytokines known to influence the migration of mononuclear effectors was upregulated, as were cytokines known to play a role in lung microbial defense and tissue repair. Another subset of cytokines associated with thrombocyte counts and function was downregulated. This study corroborates clinical findings and extends our current knowledge by providing a more comprehensive basis for the immune responses and morphology observed in laboratory and histological findings in subjects with HPS.

Materials and Methods

Subjects

Twelve clinical diagnostic serum specimens collected from 2008 to 2012 by the Nevada State Health Laboratory (NSHL) and with a confirmed diagnosis of HPS were utilized in this study. The NSHL serves as a regional reference laboratory and routinely screens subjects suspected of having HPS, by the presence of anti-hantavirus antibodies. These deidentified diagnostic specimens were deemed to be exempt from IRB approval by the University of Nevada (UNR), Research Integrity Office (Reference #616225-1) as meeting the exemption criteria defined by the Department of Health and Human Services under Human Subject Research Code 45 CFR 46.102(f). Information of each HPS case was limited to diagnosis, gender, and antibody titer range. Forty-two serum samples from healthy individuals collected under informed consent were used as controls (Human subjects protocol # B12-031). Control subjects were chosen to be consistent with published demographics of typical HPS cases regarding age and gender (male to female ratio of 54–46%, respectively, and mean age of 39.4 years) (20).

HPS Screening

Serum anti-hantavirus antibody titers were evaluated by ELISA, according to the methods described by Feldmann et al. (21). Serum dilutions (1:100–1:6400) were tested for the presence of anti-hantavirus IgG and IgM using recombinant nucleocapsid protein supplied by the United States Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). Subjects with antibody titers greater than twofold above that of negative controls were considered positive.

Multiplex Analysis

The levels of serum cytokines were analyzed using Bio-Plex (Bio-Rad, Hercules, CA, USA) multiplex magnetic bead-based antibody detection kits following the manufacturer's instructions. The Bio-Plex Pro Human Chemokine Panel (40-Plex); Bio-Plex Pro Human Th17 Cytokine Panel; Bio-Plex Pro Human Cytokine 27-plex Panel; and Bio-Plex Human Cytokine 21-plex Panel were used for analysis of a total of 68 analytes. Fifty microliters of serum from each respective case and control was analyzed using a Luminex 200 analyzer with MasterPlex CT control software and MasterPlex QT analysis software (MiraiBio, San Bruno, CA, USA). Standard curves for each analyte were generated using standards provided by manufacturer. Serum samples from HPS cases were heat inactivated and tested for the presence of infectious virus prior to Luminex analysis. The effect of heat inactivation on cytokine stability was evaluated and those that could not be normalized were excluded from analysis.

Statistical Analysis

Mann–Whitney non-parametric analysis was utilized to identify differences in medians between HPS cases and controls. In addition, we performed classification analysis using the tree-based

ensemble machine learning algorithm Random Forest (RF) (22). For this analysis, 500 random trees were built using six predictors for each node, and auto-bootstrap out-of-bag sampling was used for testing the model as previously described (23).

Results

Anti-Hantavirus Titer in HPS Serum

Twelve serum samples from subjects suspected of having hantavirus infection were tested for the presence of anti-hantavirus IgG and IgM antibodies. Antibody titers twofold greater than those of the control samples were considered diagnostic for hantavirus infection (**Table 1**). Previous reports suggest that anti-hantavirus IgM and IgG change with disease progression (24, 25). As reported by MacNeil and coworkers, early stage HPS is characterized by high IgM titers that peak within 11–14 days after onset whereas cases with early stage HPS often have no SNV-specific IgG titer (24). In contrast to IgM titers, median IgG titers typically displayed an increasing trend for a longer interval after the onset of disease. In light of the deidentified nature of our HPS cases, we used antibody titers to assess the stage of their illness. Six of our cases had high serum titer of IgM while IgG levels were low or undetectable, indicative of early stage disease. For the remaining six cases, high serum titers were observed for both IgG and IgM, consistent with late onset HPS.

Differential Expression of Serum Cytokine in HPS Cases

A total of 68 serum cytokines were measured for HPS cases and controls (**Tables 2–4**). To the best of our knowledge, 38 of these cytokines were previously uninvestigated in the context of HPS (indicated by an asterisk in **Tables 2–4**). A significant increase in the serum levels of 25 of 68 (36.7%) cytokines were observed for the HPS cases when compared to healthy controls (**Table 2**). The greatest difference was observed for IL-6, CXCL10, CX3CL1, MIF, and MIG, all of which were upregulated fivefold over those of controls ($p < 0.001$). In contrast, 15 of 68 (22.1%) cytokines were downregulated in HPS cases when compared to controls (**Table 3**), the greatest differences were observed for CXCL12, CCL21, CCL22, CCL27, and sCD40L ($p < 0.001$). Additionally, the majority of downregulated cytokines belonged to the homeostatic and inflammatory chemokine family. Of the 68 cytokines investigated, 28 (41.2%) were not statistically different when comparing cases and controls (**Table 4**).

Analysis of Serum Cytokines in Early vs. Late Stage HPS

In order to investigate the possibility that differential expression of cytokines occurs between subjects with early and late stage HPS, we compared these two subgroups with each other and to healthy controls. Surprisingly, we observed only five cytokines to be differentially expressed between the two subgroups of HPS

TABLE 1 | Antibody titer in serum from HPS cases.

Subject	IgM titer	IgG titer	Stage
1	>6400	<400	Early
2	>6400	<400	Early
3	>6400	<400	Early
4	>400	Negative	Early
5	>400	Negative	Early
6	>400	Negative	Early
7	<6400	>6400	Late
8	<6400	>6400	Late
9	<6400	>6400	Late
10	<6400	>6400	Late
11	<6400	>6400	Late
12	<6400	>6400	Late

TABLE 2 | Cytokines upregulated in HPS cases compared to healthy controls.

Analyte	Case (pg/mL), *n* = 12	Control (pg/mL), *n* = 41	*p* Value
Upregulated In HPS serum			
IL-1α	537.7 ± 95.0	179.12 ± 15.7	0.0001
IL-2RA	455.3 ± 84.6	177.3 ± 7.3	0.0001
IL-2	11.7 ± 3.6	4.7 ± 0.8	0.005
IL-3	415.1 ± 86.4	140.5 ± 11.4	0.0001
IL-6	87.9 ± 22.7	10.8 ± 2.0	0.0001
IL-10	49.2 ± 31.5	15.7 ± 1.1	0.05
IL-12(p40)	927.3 ± 175.1	280.7 ± 22.9	0.0001
IL-17A*	23.3 ± 6.8	7.5 ± 0.2	0.0001
IL-17F*	74.3 ± 19.3	17.9 ± 4.8	0.0001
IL-18*	1651.6 ± 495.1	803.6 ± 66.7	0.006
IL-22*	42.1 ± 12.1	22.7 ± 0.5	0.004
CCL23*	705.9 ± 102.5	375.7 ± 37.6	0.0004
CXCL10	2834.2 ± 913.5	197.8 ± 18.8	0.0001
CX3CL1*	1456.6 ± 321.2	241.3 ± 13.2	0.0001
GM-CSF	55.3 ± 9.7	14.2 ± 2.5	0.0001
M-CSF	4811.7 ± 167.7	415.1 ± 26.5	0.0001
VEGF	179.2 ± 122.7	48.8 ± 6.1	0.05
MIF*	4779.9 ± 2229	540.6 ± 70.5	0.001
CXCL9*	2702.7 ± 891	355.0 ± 93.0	0.0001
TNFβ	227.9 ± 26	147.9 ± 12.9	0.007
IFNα	191.9 ± 26.2	123.6 ± 9.8	0.005
LIF*	346.7 ± 40.9	216.9 ± 10.7	0.0001
b-NGF*	122.0 ± 13.8	98.3 ± 3.9	0.03
SCF*	1180.8 ± 233.9	469.3 ± 30.9	0.0001
TRAIL*	391.9 ± 82.4	266.7 ± 14.8	0.02

TABLE 3 | Cytokines downregulated in HPS cases compared to healthy controls.

Analyte	HPS (pg/mL), *n* = 12	Control (pg/mL), *n* = 41	*p* Value
Downregulated in HPS serum			
CCL1*	41.7 ± 0.3	43.3 ± 0.4	0.03
CCL5	1210.5 ± 230	5520.3 ± 670	0.001
CCL11	18.5 ± 0.9	47.1 ± 2.6	0.0001
CCL13*	37.1 ± 10.2	135.0 ± 14.1	0.0005
CCL17*	70.4 ± 31.5	241.4 ± 22.3	0.0004
CCL19*	156.2 ± 58.9	418.5 ± 38.1	0.001
CCL21*	979 ± 193	3504.6 ± 119	0.0001
CCL22*	276.2 ± 101	1112.8 ± 60.4	0.0001
CCL24*	356.7 ± 93.8	597.8 ± 49.5	0.02
CCL26*	16.4 ± 2.5	27.6 ± 1.9	0.005
CCL27*	319.8 ± 65.7	1411.4 ± 79.9	0.0001
CXCL6*	25.7 ± 44	48.2 ± 2.3	0.0002
CXCL12*	166.7 ± 32.7	2367.3 ± 104.3	0.0001
CXCL16*	183.4 ± 44.0	618.3 ± 27.9	0.0001
sCD40L	89.3 ± 54.4	2014.2 ± 128	0.0001

TABLE 4 | No significant difference in cytokine expression between HPS and healthy controls.

Analyte	HPS, *N* = 12 (pg/mL)	Healthy control, *n* = 41 (pg/mL)	*p* Value
IL-1	4.59 ± 0.1	4.8 ± 0.2	0.52
IL-1RA	93.7 ± 50.7	50.4 ± 7.4	0.15
IL-1β	2.7 ± 0.1	7.9 ± 1.9	0.1
IL-4	64.9 ± 5.2	78.4 ± 5.2	0.18
IL-5*	6.3 ± 0.6	5.9 ± 0.1	0.27
IL-7	5.3 ± 2.1	5.5 ± 0.5	0.9
IL-9*	9.9 ± 1.7	19.7 ± 11.9	0.66
IL-13	8.7 ± 0.5	8.9 ± 0.34	0.59
IL-15	9.7 ± 4.1	5.7 ± 0.07	0.07
L-16*	252.2 ± 53.4	317.2 ± 40.9	0.4
IL-21	30.7 ± 8.7	30.1 ± 6.0	0.96
IL-23	102.6 ± 25.3	95.1 ± 17.4	0.83
IL-25*	1.6 ± 0.3	2.4 ± 0.3	0.2
IL-31*	18.5 ± 3.2	21.4 ± 2.2	0.49
IL-33*	402.6 ± 125.7	723.3 ± 100.0	0.11
CCL3	18.3 ± 3.7	43.9 ± 9.5	0.15
CCL7*	196.9 ± 27	169.1 ± 23.1	0.55
CCL8	75.8 ± 11.3	95.8 ± 6.8	0.15
CXCL1*	215.9 ± 34.9	232.4 ± 13.4	0.6
CXCL2*	236.8 ± 37.7	302.1 ± 25.3	0.2
CXCL5*	1085.8 ± 230.1	798.1 ± 90.6	0.43
CXCL11*	23.5 ± 5.2	41.3 ± 10	0.35
FGF*	14.8 ± 1.3	20.7 ± 2.7	0.24
GCSF	26.2 ± 13	26.4 ± 3.3	0.98
HGF*	973.7 ± 284.6	869.9 ± 82.4	0.64
IFNγ	20.1 ± 5.0	15.4 ± 1.6	0.24
DCGF-β*	6605.8 ± 1808	4692.7 ± 353.1	0.1
PDGF	889.9 ± 302	1095.5 ± 62.1	0.29

cases (**Table 5**). Of these, median IL-33 and CXCL6 levels were greater in the early stage subjects whereas median CCL23, CXCL1, and TNF-β were greater in the late stage subjects. As expected, differences in cytokine expression between subgroups and controls were consistent with differences observed between total HPS cases and controls (data not shown).

Classification of Cytokines by Importance

Given the complex interactions of cytokines with immune and non-immune cells, clarification of how distinct cytokines contribute to a pathological situation is often difficult to resolve. In order to provide insight into this issue, we implemented the machine logic algorithm RF to analyze our data set and potentially identify the most important cytokines that define this disease. For our analysis, 500 random decision trees were constructed with six predictors at each node, and auto-bootstrap out-of-bag sampling was implemented to test the accuracy of model. This model accurately identified HPS cases with 100% specificity and 73.81% sensitivity (**Table 6**). The 10 most significant cytokines for delineating HPS in decreasing order of importance are: M-CSF, CXCL16, sCD40, CXCL12, CCL22, IL-1a, CCL21, IL-12p40, CCL17, and IL-1b.

Discussion

The microvascular endothelium is principal target of hantavirus infection in humans and its infection in lung tissue results in significant pathology (26). Infection of endothelial cells leads to increased vascular permeability without an observable cytopathic effect; therefore, the pathogenesis of HPS is likely indirect with immune responses, such as cytokine production, playing an important role. The cytokines that we observed to be upregulated in the serum of HPS cases are involved in a number of antiviral defense mechanisms including proliferation, maturation, and activation of leukocytes, as well as survival of leukocytes, and regulation of endothelial monolayer permeability (**Table 2**). High levels of IL-1α, IL-6, MIF, and TNF-β suggest a strong proinflammatory milieu in the serum of HPS cases, thus promoting both inflammation and activation of immune responses. We also observed stem cell proliferation factors to be upregulated, potentially promoting the proliferation and differentiation of subsets of immune effector cells. For example, proliferation of myeloid progenitors is strongly supported by IL-3, GM-CSF, and M-CSF. Increased serum concentrations of GM-CSF and M-CSF also suggest proliferation of monocytes and granulocytes (neutrophils, eosinophils, and basophils). Upregulation of the pluripotent factor, SCF, was also observed in association with HPS, suggesting increased proliferation of T lymphocytes, NK cells, and dendritic cells.

We observed a subset of 15 serum cytokines to be downregulated in our HPS cases (**Table 4**). Twelve of these cytokines are involved in chemotaxis of lymphocytes, such as B cells, T cells, and NK cells, to sites of infection. Some of these cytokines, including CCL22, CXCL12, and CCL17, are associated with activation of Th2-type immunity and are potent recruiters of Th2 cells to the lungs, as well as activators of pre-B cells (27–29). A number of cytokines identified as differentially expressed in the present study are consistent with putative immune responses of lung tissue. For example, we observed the upregulation of serum IL-17F, CXCL16, and IL-22, which are involved in the regulation of leukocyte migration into lung tissue, as well as lung tissue repair (30–33). Upregulation of IL-17F has also been observed in the lung tissue of asthmatic cases and its level positively correlated with disease severity (30, 34, 35). Overexpression of IL-17F promotes neutrophil infiltration and increased airways sensitivity and thus has a significant impact on lung function (35). IL-22 is considered a key cytokine for mucosal tissue repair (36) and by activating antimicrobial responses in lung epithelial cells; it has been shown to be critical for host defense as well. Also, IL-22 promotes lung epithelial cell proliferation (37) and therefore, based on our analyses, the cytokine profile observed in our HPS cases is consistent with a pulmonary antimicrobial response and subsequent mononuclear cell migration into the lung.

The serum cytokine profile observed in our HPS subjects also suggests a mobilization of mononuclear immune effector cells (**Table 2**). IL-12(p40) is an autocrine chemoattractant released by activated macrophages and promotes Th1-type immunity (38, 39). Additionally, serum levels for several potent T lymphocyte and NK chemoattractants were upregulated, including CXCL10, MIG, and CCL23 (15, 16, 40, 41). MIF and VEGF, which are regulators of mononuclear cell transendothelial migration, were upregulated as well. Migration of leukocytes can also be facilitated by the upregulation of adhesion molecules on the surface of endothelial cells in response to VEGF, IL-1α, and IL-6 (42, 43).

TABLE 5 | Serum cytokine profile during early and late stages of HPS.

Analyte	HPS early (pg/mL)	HPS late (pg/nL)	Control (pg/mL)	*p* Value*	*p* Value**	*p* Value***
IL-1α	316.3 ± 60.4	545 ± 98.1	179.12 ± 15.7	0.006	0.0001	
IL-2RA	345.5 ± 92.5	454.9 ± 44.2	177.3 ± 7.3	0.001	0.0001	
IL-2	11.9 ± 5.5	10.1 ± 4.6	4.7 ± 0.8		0.02	
IL-3	269.3 ± 39.8	331.6 ± 87.2	140.5 ± 11.4	0.0004	0.0002	
IL-6	50.4 ± 30.5	109.9 ± 25.9	10.8 ± 2.0	0.003	0.0001	
IL-10	34.6 ± 12.4	14.6 ± 0.5	15.7 ± 1.1		0.02	
IL-12(p40)	613.9 ± 77.3	815.1 ± 196.3	280.7 ± 22.9	0.0001	0.0001	
IL-15	13.8 ± 7.5	5.6 ± 0.4	5.7 ± 4.1	0.008		
IL-17A	20.1 ± 6.2	24.6 ± 10.5	7.5 ± 0.2	0.0001	0.001	
IL-17F	61.0 ± 24.8	52.7 ± 10.7	17.9 ± 4.8	0.009	0.03	
IL-22	32.2 ± 12.5	55.8 ± 24.4	22.7 ± 0.5	0.0005		
IL-33	651.5 ± 179.2	74.5 ± 19.3	723.3 ± 100			0.03
CCL5	1339.8 ± 409.3	1168.7 ± 182.5	5520.3 ± 670	0.02	0.05	
CCL11	20.0 ± 1.3	17.5 ± 0.6	47.1 ± 2.6	0.0002	0.0009	
CCL17	124.1 ± 51.5	17.7 ± 5.6	241.4 ± 22.3		0.003	
CCL19	250.1 ± 64.4	126.3 ± 32.9	319.6 ± 38.1		0.02	
CCL21	732.9 ± 136.8	1185.5 ± 303.1	3504.6 ± 119	2.8E-11	0.0001	
CCL22	458.6 ± 159.4	108.5 ± 39.9	1112.8 ± 60.4	0.0004	0.0001	
CCL23	489.8 ± 112.4	990.7 ± 122.7	375.7 ± 37.6		0.0001	0.02
CCL24	229.5 ± 53.3	608.2 ± 180.2	597.8 ± 49.5		0.007	
CCL26	18.5 ± 4.3	13.9 ± 2.6	27.6 ± 1.9		0.03	
CCL27	306.4 ± 91.9	391.2 ± 97.1	1411.4 ± 79.9	0.0001	0.0003	
CXCL1	470.0 ± 50.8	954.3 ± 176.8	232.4 ± 13.4		0.03	0.01
CXCL5	2005.6 ± 1072.8	225.0 ± 49.9	708.1 ± 90.6	0.02		
CXCL6	32.9 ± 8.3	21.3 ± 10.9	48.2 ± 2.3		0.04	0.003
CXCL10	2785.2 ± 146.2	3843.1 ± 1266	197.8 ± 18.8	0.0001	0.0001	
CXCL12	191.1 ± 47.2	181.0 ± 42.6	2367.3 ± 104.3	0.0001	0.0001	
CXCL16	201.5 ± 73.8	175.2 ± 29.9	618.1 ± 27.9	0.0001	0.0001	
CX3CL1	1020.4 ± 440.6	1710.3 ± 309.1	241.3 ± 13.2	0.0001	0.0001	
GM-CSF	67.3 ± 9.7	43.6 ± 16.6	14.2 ± 2.5	0.0001	0.005	
DCGF-β	4611 ± 1036.1	11215.6 ± 3269	4692.7 ± 353.1		0.0002	
LIF	253.3 ± 39.1	386.8 ± 50.4	216.9 ± 10.7		0.0001	
M-CSF	1721.7 ± 475.2	3563.8 ± 1221.1	415.1 ± 26.5	0.0001	0.0001	
MIG	2924.6 ± 1596.1	3152.6 ± 2746.3	355.0 ± 93.0	0.0003	0.0001	
MIF	1977.3 ± 540.6	666.2 ± 200.9	540.6 ± 70.5	0.008	0.0001	
sCD40L	157.8 ± 85.1	15.7 ± 4.3	2014.2 ± 128	0.0001	0.0001	
SCF	798.8 ± 207.6	1390.8 ± 443.1	469.3 ± 30.9	0.006	0.0001	
TNFβ	167 ± 16.4	226.9 ± 16.1	147.9 ± 12.9			0.04
VEGF	286.5 ± 48.8	101.4 ± 28.5	48.86.1	0.01	0.02	

**p value early phase to control; **p value late phase to control; ***p value early to late phase.*

MIF and VEGF promote expression of the adhesion molecules, E-selectin, ICAM-1, and VCAM-1, and increase vascular permeability (44, 45). Additionally, VEGF can decrease tight junctions between endothelial cells enabling transmigration of immune effector cells (42, 46). The observed increased serum levels of CXCL1, which may lead to release of VEGF-A from hantavirus-activated endothelial cells, further suggests that upregulation of VEGF plays a role in HPS (47, 48).

Cytokines including CXCL10, MIF, MIG, IL-12(p40), IL-17A, and CCL23 are known to promote proliferation and migration of mononuclear immune cells, such as T lymphocytes, NK cells, monocytes, and dendritic cells (15, 49–51). Consequently, our data support the previous observations of others whereby mononuclear cell and immunoblasts are the principal cellular infiltrate in the lungs of HPS cases (12). Nevertheless, the observed cytokine expression also is consistent with the activation and migration of neutrophils. Previous studies suggest that the cytokines, IL-17F, VEGF, CXCL1, GM-CSF, and IL-22, promote neutrophil migration and lung tissue repair (52–54). These data corroborate a previous report by Mori et al., who observed low-level neutrophil infiltration in the lungs of HPS case (12). Interestingly, serum level of CXCL8, the prototype neutrophil chemoattractant, was not significantly elevated in the HPS cases in our study; however, it was identified as one of the top 10 cytokines by our RF analysis, suggesting its expression, or lack thereof, plays an important role in HPS pathology. Our data further suggest that a Th17 shift occurs in HPS (55). In the presence of IL-23, non-Th17 cells can produce IL-17 (56); however, we observed no differential expression of serum IL-23 in HPS cases. Therefore, it is likely that activated Th17 lymphocytes were the source of IL-17 in the serum of our HPS cases.

Expression of IL-17 and IL-22 in HPS suggests a developing antimicrobial state in the lung. It has been reported that IL-17 and IL-22 activate β-defensins and the S100 family of proteins (52, 57). *In vivo* studies using knockout mice have demonstrated that IL-17 and IL-22 are crucial for bacterial defense in the lung (58, 59). Furthermore, it has been reported that IL-17R signaling is mandatory for the establishment of an antibacterial response

TABLE 6 | Random forest analysis of serum cytokines in HPS vs. controls.

Variable	Score (%)	Changes in HPS serum	Variable	Score (%)	Changes in HPS serum
M-CSF	100.0000	Upregulated	IL-17F	19.4733	Upregulated
CXCL16	98.7888	Downregulated	CCL3	19.1369	Unchanged
sCD40L	96.8968	Downregulated	CCL1	18.8358	Downregulated
CXCL12	85.5322	Downregulated	CXCL11	18.5085	Unchanged
CCL22	78.4301	Downregulated	DCGFB	17.9953	Unchanged
IL-1α	74.0061	Upregulated	IL-4	17.7238	Unchanged
CCL21	70.3732	Downregulated	IL-25	17.4077	Unchanged
IL-12(p40)	62.9938	Upregulated	IL-33	16.6915	Unchanged
CCL17	62.8689	Downregulated	CCL7	16.5016	Unchanged
IL-1β	61.4314	Unchanged	TRAIL	15.7741	Upregulated
CCL5	61.0088	Downregulated	IL-9	14.3617	Unchanged
IL-3	58.5351	Upregulated	IL-18	12.0276	Upregulated
CCL13	58.0210	Downregulated	IL-7	10.5668	Unchanged
CXCL9	52.4830	Upregulated	IL-22	10.2808	Upregulated
CXCL10	50.3664	Upregulated	CXCL2	9.3174	Unchanged
CCL11	48.5759	Downregulated	MIF	8.8718	Upregulated
CCL27	46.5450	Downregulated	IL-16	8.8434	Unchanged
CXCL5	46.0115	Unchanged	b-NGF	7.9004	Upregulated
CX3CL1	45.6097	Upregulated	IL-31	7.0565	Unchanged
GM-CSF	43.1944	Upregulated	IL-10	6.3552	Upregulated
IFNα	41.2777	Upregulated	CCL8	6.1227	Unchanged
LIF	40.7034	Upregulated	IL-17A	5.3501	Upregulated
CCL24	39.4832	Downregulated	INFG	4.9877	Unchanged
IL-2RA	38.9993	Upregulated	GCSF	4.4335	Upregulated
PDGF	36.2886	Unchanged	IL-1	4.1642	Unchanged
CCL19	34.6337	Downregulated	FGF	4.0244	Unchanged
IL-6	31.5406	Upregulated	HGF	3.9312	Unchanged
CXCL6	30.4604	Downregulated	IL-1RA	3.5669	Unchanged
IL-15	25.5225	Unchanged	CXCL1	3.3770	Unchanged
TNFβ	24.6500	Upregulated	IL-5	2.6487	Unchanged
SCF	24.2768	Upregulated	IL-23	2.2930	Unchanged
IL-2	22.9123	Upregulated	VEGF	0.9825	Upregulated
CCL26	21.2675	Downregulated	IL-13	0.0038	Unchanged
CCL23	20.7866	Upregulated			

to *M. pneumoniae*, systemic fungal infection, *B. fragilis*, and *E. coli* (60–63). Consistent with this statement, a protective role for IL-22 was recently reported for experimental influenza A virus infection (64).

We also observed a subset of cytokines involved in the regulation of platelet counts and function to be downregulated in the serum of our HPS subjects, including sCD40L, CCL5, CCL22, and CXCL12 (**Table 3**). Consistent with our observations and the pathophysiology of HPS, CXCL12 and CCL22 act on platelets to rapidly stimulate their adhesion (65), and CCL5 and sCD40L are released by activated platelets (66–68). Wenzel and coworkers reported that serum levels of sCD40L closely correlate with platelets counts and that they are increased upon thrombocyte transfusion (69). Viallard et al. also reported a correlation between thrombocyte counts and serum sCD40L, implying that it may be used as a surrogate marker for platelet counts (66). Decreased thrombocyte counts are also well documented in association with HPS (2, 70) and our observation of downregulated sCD40L presents a potential biomarker for the thrombocytopenia. Notwithstanding, decreased serum CCL22 might also reflect the development of the thrombocytopenia observed in HPS cases. It has been shown that CCL22 is capable of aggregating platelets in the presence of low concentrations of thrombin or adenosine diphosphate (ADP), and can rapidly stimulate platelets adhesion (65). It is noteworthy that endothelial cells do not produce this cytokine; dendritic cells are the main source of CCL22 (71). Therefore, the thrombocyte aggregation and depletion observed in HPS may be the result of cytokine-driven immune responses.

Serum levels of CCL21 and CCL27 were also downregulated in the serum of our HPS subjects. These cytokines have tissue-specific activity; for example, CCL21 orchestrates dendritic cell and T cell trafficking to the lymph nodes (72–74) and CCL27 regulates migration of immune effector cells to the skin (75). Taken together, these findings suggest that the cytokines expressed during HPS promote lung tissue infiltration while reducing leukocyte trafficking to other organs and tissues.

In order to investigate the contribution of each respective cytokine to the disease process, we conducted classification analysis by RF. Of the 10 most important cytokines identified by this analysis, 3 were significantly upregulated, as determined by Mann–Whitney analysis; however, we also observed 6 to be downregulated. This observation underscores the importance of cytokine inhibition in the disease process and further suggests that depressed serum cytokine expression may be an important biomarker for monitoring disease progression.

Overall, the majority of downregulated serum cytokines were associated with Th2-type immune activation; these included CCL21, CCL17, CCL13, and CCL11. Furthermore, the cytokines

significantly upregulated in HPS cases were those promoting Th1-type immunity; these included CXCL9, CXCL10, and IL-12(p40). The cytokines M-CSF, CXCL12, IL-3, LIF, GM-CSF, CCL24, which facilitate activation, differentiation, and bone marrow mobilization of myeloid progenitors, were also identified by RF analysis to differentiate HPS cases from controls. RF analysis further identified chemokines associated with platelet aggregation as important in differentiating cases from controls. Interestingly, sCD40L and CXCL12 were ranked, respectively, as the third and fourth most import cytokine in our RF analysis. Chemokines, such as sCD40L, CXCL12, and CCL17, which are stored in platelet granules, are released upon platelet aggregation, a process that is critical in HPS pathology (2, 76–78). Accordingly, nadir platelet counts in HPS may explain low serum CXCL1, CCL17, and sCD40. Taken together, RF analysis supports the supposition that HPS pathogenesis may be characterized by Th1-type immune responses and thrombocytopenia.

In summary, our data suggest that HPS is characterized by a serum cytokine profile that is consistent with putative immune responses in lung tissue. Strong activation of mononuclear immune effectors including T lymphocytes, NK cells, and dendritic cells is also suggested by this cytokine profile. Additionally, our data imply that decreased counts and increased aggregation of thrombocytes in HPS might be explained in part by the immune response to viral infection. Lastly, to the best of our knowledge, our data provide the first evidence of Th17 lymphocyte activation in association with HPS. The data presented in this study are suggestive of putative *in vivo* immune mechanisms and may identify the role of these cytokines in HPS pathophysiology; however, future studies using animal models would be necessary to definitively confirm their involvement.

Acknowledgments

An award to VL from the Department of Defense (DOD) grant PR131133 supported these studies. A fellowship provided by the Program of Competitive Growth of Kazan Federal University supported the work of AR.

References

1. Duchin JS, Koster FT, Peters CJ, Simpson GL, Tempest B, Zaki SR, et al. Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. The Hantavirus Study Group. *N Engl J Med* (1994) **330**(14):949–55. doi:10.1056/NEJM199404073301401
2. Nolte KB, Feddersen RM, Foucar K, Zaki SR, Koster FT, Madar D, et al. Hantavirus pulmonary syndrome in the United States: a pathological description of a disease caused by a new agent. *Hum Pathol* (1995) **26**(1):110–20. doi:10.1016/0046-8177(95)90123-X
3. Hjelle B, Lee SW, Song W, Torrez-Martinez N, Song JW, Yanagihara R, et al. Molecular linkage of hantavirus pulmonary syndrome to the white-footed mouse, *Peromyscus leucopus*: genetic characterization of the M genome of New York virus. *J Virol* (1995) **69**(12):8137–41.
4. Khan AS, Spiropoulou CF, Morzunov S, Zaki SR, Kohn MA, Nawas SR, et al. Fatal illness associated with a new hantavirus in Louisiana. *J Med Virol* (1995) **46**(3):281–6. doi:10.1002/jmv.1890460320
5. Lopez N, Padula P, Rossi C, Lazaro ME, Franze-Fernandez MT. Genetic identification of a new hantavirus causing severe pulmonary syndrome in Argentina. *Virology* (1996) **220**(1):223–6. doi:10.1006/viro.1996.0305
6. Hjelle B, Jenison S, Mertz G, Koster F, Foucar K. Emergence of hantaviral disease in the southwestern United States. *West J Med* (1994) **161**(5):467–73.
7. Zaki SR, Khan AS, Goodman RA, Armstrong LR, Greer PW, Coffield LM, et al. Retrospective diagnosis of hantavirus pulmonary syndrome, 1978-1993: implications for emerging infectious diseases. *Arch Pathol Lab Med* (1996) **120**(2):134–9.
8. Centers for Disease Control and Prevention. *U.S. HPS Cases, by Reporting State*. Atlanta, GA: Centers for Disease Control and Prevention (2014).
9. Hartline J, Mierek C, Knutson T, Kang C. Hantavirus infection in North America: a clinical review. *Am J Emerg Med* (2013) **31**(6):978–82. doi:10.1016/j.ajem.2013.02.001
10. Warner GS. Hantavirus illness in humans: review and update. *South Med J* (1996) **89**(3):264–71. doi:10.1097/00007611-199603000-00002
11. Hallin GW, Simpson SQ, Crowell RE, James DS, Koster FT, Mertz GJ, et al. Cardiopulmonary manifestations of hantavirus pulmonary syndrome. *Crit Care Med* (1996) **24**(2):252–8. doi:10.1097/00003246-199602000-00012
12. Mori M, Rothman AL, Kurane I, Montoya JM, Nolte KB, Norman JE, et al. High levels of cytokine-producing cells in the lung tissues of patients with fatal hantavirus pulmonary syndrome. *J Infect Dis* (1999) **179**(2):295–302. doi:10.1086/314597
13. Khaiboullina SF, Rizvanov AA, Otteson E, Miyazato A, Maciejewski J, St Jeor S. Regulation of cellular gene expression in endothelial cells by Sin Nombre and prospect hill viruses. *Viral Immunol* (2004) **17**(2):234–51. doi:10.1089/0882824041310504
14. Sundstrom JB, McMullan LK, Spiropoulou CF, Hooper WC, Ansari AA, Peters CJ, et al. Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells. *J Virol* (2001) **75**(13):6070–85. doi:10.1128/JVI.75.13.6070-6085.2001
15. Moser B, Loetscher M, Piali L, Loetscher P. Lymphocyte responses to chemokines. *Int Rev Immunol* (1998) **16**(3–4):323–44. doi:10.3109/08830189809043000
16. Graziano FM, Cook EB, Stahl JL. Cytokines, chemokines, RANTES, and eotaxin. *Allergy Asthma Proc* (1999) **20**(3):141–6. doi:10.2500/108854199778553055
17. Prescott J, Ye C, Sen G, Hjelle B. Induction of innate immune response genes by Sin Nombre hantavirus does not require viral replication. *J Virol* (2005) **79**(24):15007–15. doi:10.1128/JVI.79.24.15007-15015.2005
18. Kraus AA, Raftery MJ, Giese T, Ulrich R, Zawatzky R, Hippenstiel S, et al. Differential antiviral response of endothelial cells after infection with pathogenic and nonpathogenic hantaviruses. *J Virol* (2004) **78**(12):6143–50. doi:10.1128/JVI.78.12.6143-6150.2004
19. Borges AA, Campos GM, Moreli ML, Moro Souza RL, Saggioro FP, Figueiredo GG, et al. Role of mixed Th1 and Th2 serum cytokines on pathogenesis and prognosis of hantavirus pulmonary syndrome. *Microbes Infect* (2008) **10**(10–11):1150–7. doi:10.1016/j.micinf.2008.06.006
20. Khan AS, Khabbaz RF, Armstrong LR, Holman RC, Bauer SP, Graber J, et al. Hantavirus pulmonary syndrome: the first 100 US cases. *J Infect Dis* (1996) **173**(6):1297–303. doi:10.1093/infdis/173.6.1297
21. Feldmann H, Sanchez A, Morzunov S, Spiropoulou CF, Rollin PE, Ksiazek TG, et al. Utilization of autopsy RNA for the synthesis of the nucleocapsid antigen of a newly recognized virus associated with hantavirus pulmonary syndrome. *Virus Res* (1993) **30**(3):351–67. doi:10.1016/0168-1702(93)90101-R
22. Breiman L. *Classification and Regression Trees*. Belmont, CA: Wadsworth International Group (1984).
23. Khaiboullina SF, DeMeirleir KL, Rawat S, Berk GS, Gaynor-Berk RS, Mijatovic T, et al. Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis. *Cytokine* (2015) **72**(1):1–8. doi:10.1016/j.cyto.2014.11.019
24. MacNeil A, Comer JA, Ksiazek TG, Rollin PE. Sin Nombre virus-specific immunoglobulin M and G kinetics in hantavirus pulmonary syndrome and the role played by serologic responses in predicting disease outcome. *J Infect Dis* (2010) **202**(2):242–6. doi:10.1086/653482
25. Padula PJ, Colavecchia SB, Martinez VP, Gonzalez Della Valle MO, Edelstein A, Miguel SD, et al. Genetic diversity, distribution, and serological features of hantavirus infection in five countries in South America. *J Clin Microbiol* (2000) **38**(8):3029–35.
26. Zaki SR, Greer PW, Coffield LM, Goldsmith CS, Nolte KB, Foucar K, et al. Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. *Am J Pathol* (1995) **146**(3):552–79.

27. Weiss JM, Cufi P, Bismuth J, Eymard B, Fadel E, Berrih-Aknin S, et al. SDF-1/CXCL12 recruits B cells and antigen-presenting cells to the thymus of autoimmune myasthenia gravis patients. *Immunobiology* (2013) **218**(3):373–81. doi:10.1016/j.imbio.2012.05.006
28. Nagasawa T, Tachibana K, Kishimoto T. A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Semin Immunol* (1998) **10**(3):179–85. doi:10.1006/smim.1998.0128
29. Nakayama T, Hieshima K, Nagakubo D, Sato E, Nakayama M, Kawa K, et al. Selective induction of Th2-attracting chemokines CCL17 and CCL22 in human B cells by latent membrane protein 1 of Epstein-Barr virus. *J Virol* (2004) **78**(4):1665–74. doi:10.1128/JVI.78.4.1665-1674.2004
30. Fujie H, Niu K, Ohba M, Tomioka Y, Kitazawa H, Nagashima K, et al. A distinct regulatory role of Th17 cytokines IL-17A and IL-17F in chemokine secretion from lung microvascular endothelial cells. *Inflammation* (2012) **35**(3):1119–31. doi:10.1007/s10753-011-9419-0
31. Al-Ramli W, Prefontaine D, Chouiali F, Martin JG, Olivenstein R, Lemiere C, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* (2009) **123**(5):1185–7. doi:10.1016/j.jaci.2009.02.024
32. Morgan AJ, Guillen C, Symon FA, Huynh TT, Berry MA, Entwisle JJ, et al. Expression of CXCR6 and its ligand CXCL16 in the lung in health and disease. *Clin Exp Allergy* (2005) **35**(12):1572–80. doi:10.1111/j.1365-2222.2005.02383.x
33. Ivanov S, Renneson J, Fontaine J, Barthelemy A, Paget C, Fernandez EM, et al. Interleukin-22 reduces lung inflammation during influenza A virus infection and protects against secondary bacterial infection. *J Virol* (2013) **87**(12):6911–24. doi:10.1128/JVI.02943-12
34. Kawaguchi M, Kokubu F, Huang SK, Homma T, Odaka M, Watanabe S, et al. The IL-17F signaling pathway is involved in the induction of IFN-gamma-inducible protein 10 in bronchial epithelial cells. *J Allergy Clin Immunol* (2007) **119**(6):1408–14. doi:10.1016/j.jaci.2007.02.036
35. Hizawa N, Kawaguchi M, Huang SK, Nishimura M. Role of interleukin-17F in chronic inflammatory and allergic lung disease. *Clin Exp Allergy* (2006) **36**(9):1109–14. doi:10.1111/j.1365-2222.2006.02550.x
36. Rutz S, Eidenschenk C, Ouyang W. IL-22, not simply a Th17 cytokine. *Immunol Rev* (2013) **252**(1):116–32. doi:10.1111/imr.12027
37. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* (2008) **14**(3):275–81. doi:10.1038/nm1710
38. Bastos KR, Alvarez JM, Marinho CR, Rizzo LV, Lima MR. Macrophages from IL-12p40-deficient mice have a bias toward the M2 activation profile. *J Leukoc Biol* (2002) **71**(2):271–8.
39. Cooper AM, Khader SA. IL-12p40: an inherently agonistic cytokine. *Trends Immunol* (2007) **28**(1):33–8. doi:10.1016/j.it.2006.11.002
40. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* (2011) **89**(2):207–15. doi:10.1038/icb.2010.158
41. Broxmeyer HE, Kim CH, Cooper SH, Hangoc G, Hromas R, Pelus LM. Effects of CC, CXC, C, and CX3C chemokines on proliferation of myeloid progenitor cells, and insights into SDF-1-induced chemotaxis of progenitors. *Ann N Y Acad Sci* (1999) **872**:142–62.
42. Davis B, Tang J, Zhang L, Mu D, Jiang X, Biran V, et al. Role of vasodilator stimulated phosphoprotein in VEGF induced blood-brain barrier permeability in endothelial cell monolayers. *Int J Dev Neurosci* (2010) **28**(6):423–8. doi:10.1016/j.ijdevneu.2010.06.010
43. Deissler H, Deissler H, Lang S, Lang GE. VEGF-induced effects on proliferation, migration and tight junctions are restored by ranibizumab (Lucentis) in microvascular retinal endothelial cells. *Br J Ophthalmol* (2008) **92**(6):839–43. doi:10.1136/bjo.2007.135640
44. Cheng Q, McKeown SJ, Santos L, Santiago FS, Khachigian LM, Morand EF, et al. Macrophage migration inhibitory factor increases leukocyte-endothelial interactions in human endothelial cells via promotion of expression of adhesion molecules. *J Immunol* (2010) **185**(2):1238–47. doi:10.4049/jimmunol.0904104
45. Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. Regulation of transendothelial migration of hematopoietic progenitor cells. *Ann N Y Acad Sci* (1999) **872**:176–85. doi:10.1111/j.1749-6632.1999.tb08463.x
46. Deissler HL, Deissler H, Lang GE. Inhibition of vascular endothelial growth factor (VEGF) is sufficient to completely restore barrier malfunction induced by growth factors in microvascular retinal endothelial cells. *Br J Ophthalmol* (2011) **95**(8):1151–6. doi:10.1136/bjo.2010.192229
47. Scapini P, Morini M, Tecchio C, Minghelli S, Di Carlo E, Tanghetti E, et al. CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. *J Immunol* (2004) **172**(8):5034–40. doi:10.4049/jimmunol.172.8.5034
48. DiStasi MR, Ley K. Opening the flood-gates: how neutrophil-endothelial interactions regulate permeability. *Trends Immunol* (2009) **30**(11):547–56. doi:10.1016/j.it.2009.07.012
49. Piali L, Weber C, LaRosa G, Mackay CR, Springer TA, Clark-Lewis I, et al. The chemokine receptor CXCR3 mediates rapid and shear-resistant adhesion-induction of effector T lymphocytes by the chemokines IP10 and Mig. *Eur J Immunol* (1998) **28**(3):961–72. doi:10.1002/(SICI)1521-4141(199803)28:03<961::AID-IMMU961>3.0.CO;2-4
50. Khader SA, Partida-Sanchez S, Bell G, Jelley-Gibbs DM, Swain S, Pearl JE, et al. Interleukin 12p40 is required for dendritic cell migration and T cell priming after *Mycobacterium tuberculosis* infection. *J Exp Med* (2006) **203**(7):1805–15. doi:10.1084/jem.20052545
51. Shahrara S, Pickens SR, Mandelin AM II, Karpus WJ, Huang Q, Kolls JK, et al. IL-17-mediated monocyte migration occurs partially through CC chemokine ligand 2/monocyte chemoattractant protein-1 induction. *J Immunol* (2010) **184**(8):4479–87. doi:10.4049/jimmunol.0901942
52. Khader SA, Gaffen SL, Kolls JK. Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal Immunol* (2009) **2**(5):403–11. doi:10.1038/mi.2009.100
53. Laan M, Cui ZH, Hoshino H, Lotvall J, Sjostrand M, Gruenert DC, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* (1999) **162**(4):2347–52.
54. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* (2001) **194**(4):519–27. doi:10.1084/jem.194.4.519
55. Chen Z, O'Shea JJ. Th17 cells: a new fate for differentiating helper T cells. *Immunol Res* (2008) **41**(2):87–102. doi:10.1007/s12026-007-8014-9
56. McAleer JP, Kolls JK. Mechanisms controlling Th17 cytokine expression and host defense. *J Leukoc Biol* (2011) **90**(2):263–70. doi:10.1189/jlb.0211099
57. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* (2006) **203**(10):2271–9. doi:10.1084/jem.20061308
58. Berger T, Togawa A, Duncan GS, Elia AJ, You-Ten A, Wakeham A, et al. Lipocalin 2-deficient mice exhibit increased sensitivity to *Escherichia coli* infection but not to ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* (2006) **103**(6):1834–9. doi:10.1073/pnas.0510847103
59. Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* (2004) **432**(7019):917–21. doi:10.1038/nature03104
60. Wu Q, Martin RJ, Rino JG, Breed R, Torres RM, Chu HW. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory Mycoplasma pneumoniae infection. *Microbes Infect* (2007) **9**(1):78–86. doi:10.1016/j.micinf.2006.10.012
61. Huang W, Na L, Fidel PL, Schwarzenberger P. Requirement of interleukin-17A for systemic anti-*Candida albicans* host defense in mice. *J Infect Dis* (2004) **190**(3):624–31. doi:10.1086/422329
62. Chung DR, Kasper DL, Panzo RJ, Chitnis T, Grusby MJ, Sayegh MH, et al. CD4+ T cells mediate abscess formation in intra-abdominal sepsis by an IL-17-dependent mechanism. *J Immunol* (2003) **170**(4):1958–63. doi:10.4049/jimmunol.170.4.1958
63. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J Immunol* (2007) **178**(7):4466–72. doi:10.4049/jimmunol.178.7.4466
64. Paget C, Ivanov S, Fontaine J, Renneson J, Blanc F, Pichavant M, et al. Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection: potential role in protection against lung epithelial damages. *J Biol Chem* (2012) **287**(12):8816–29. doi:10.1074/jbc.M111.304758
65. Gear AR, Suttitanamongkol S, Viisoreanu D, Polanowska-Grabowska RK, Raha S, Camerini D. Adenosine diphosphate strongly potentiates the ability of the chemokines MDC, TARC, and SDF-1 to stimulate platelet function. *Blood* (2001) **97**(4):937–45. doi:10.1182/blood.V97.4.937

66. Viallard JF, Solanilla A, Gauthier B, Contin C, Dechanet J, Grosset C, et al. Increased soluble and platelet-associated CD40 ligand in essential thrombocythemia and reactive thrombocytosis. *Blood* (2002) **99**(7):2612–4. doi:10.1182/blood.V99.7.2612
67. Kameyoshi Y, Dorschner A, Mallet AI, Christophers E, Schroder JM. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med* (1992) **176**(2):587–92. doi:10.1084/jem.176.2.587
68. Antczak AJ, Singh N, Gay SR, Worth RG. IgG-complex stimulated platelets: a source of sCD40L and RANTES in initiation of inflammatory cascade. *Cell Immunol* (2010) **263**(1):129–33. doi:10.1016/j.cellimm.2010.03.009
69. Wenzel F, Gunther W, Baertl A, Gruber W, Sorg RV, Haas R, et al. Platelet transfusion alters CD40L blood level and release capacity in patients suffering from thrombocytopenia. *Transfusion* (2012) **52**(6):1213–20. doi:10.1111/j.1537-2995.2011.03438.x
70. Sargianou M, Watson DC, Chra P, Papa A, Starakis I, Gogos C, et al. Hantavirus infections for the clinician: from case presentation to diagnosis and treatment. *Crit Rev Microbiol* (2012) **38**(4):317–29. doi:10.3109/1040841X.2012.673553
71. Vulcano M, Albanesi C, Stoppacciaro A, Bagnati R, D'Amico G, Struyf S, et al. Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. *Eur J Immunol* (2001) **31**(3):812–22. doi:10.1002/1521-4141(200103)31:3<812::AID-IMMU812>3.0.CO;2-L
72. Foo SY, Phipps S. Regulation of inducible BALT formation and contribution to immunity and pathology. *Mucosal Immunol* (2010) **3**(6):537–44. doi:10.1038/mi.2010.52
73. Johnson LA, Jackson DG. Cell traffic and the lymphatic endothelium. *Ann N Y Acad Sci* (2008) **1131**:119–33. doi:10.1196/annals.1413.011
74. Johnson LA, Jackson DG. Control of dendritic cell trafficking in lymphatics by chemokines. *Angiogenesis* (2014) **17**(2):335–45. doi:10.1007/s10456-013-9407-0
75. Kunkel EJ, Butcher EC. Chemokines and the tissue-specific migration of lymphocytes. *Immunity* (2002) **16**(1):1–4. doi:10.1016/S1074-7613(01)00261-8
76. von Hundelshausen P, Weber C. Platelets as immune cells: bridging inflammation and cardiovascular disease. *Circ Res* (2007) **100**(1):27–40. doi:10.1161/01.RES.0000252802.25497.b7
77. Andre P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. *Circulation* (2002) **106**(8):896–9. doi:10.1161/01.CIR.0000028962.04520.01
78. Knust B, Macneil A, Rollin PE. Hantavirus pulmonary syndrome clinical findings: evaluating a surveillance case definition. *Vector Borne Zoonotic Dis* (2012) **12**(5):393–9. doi:10.1089/vbz.2011.0764

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DOI: 10.1111/eci.12363

REVIEW

Plasmacytoid dendritic cells, a role in neoplastic prevention and progression

Vincent C. Lombardi*,†, Svetlana F. Khaiboullina*,† and Albert A. Rizvanov†

*Department of Biochemistry and Molecular Biology, University of Nevada School of Medicine, University of Nevada, Reno, NV, USA, †Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Republic of Tatarstan, Russian Federation

ABSTRACT

Background Plasmacytoid dendritic cells (pDCs) are multifunctional bone-marrow-derived immune cells that are key players in bridging the innate and adaptive immune systems. Activation of pDCs through toll-like receptor agonists has proven to be an effective treatment for some neoplastic disorders.

Materials and methods In this mini-review, we will explore the fascinating contribution of pDCs to neoplastic pathology and discuss their potential utilization in cancer immunotherapy.

Results Current research suggests that pDCs have cytotoxic potential and can effectively induce apoptosis of tumour-derived cells lines. They are also reported to display tolerogenic function with the ability to suppress T-cell proliferation, analogous to regulatory T cells. In this capacity, they are critical in the suppression of autoimmunity but can be exploited by tumour cells to circumvent the expansion of tumour-specific T cells, thereby allowing tumours to persist.

Conclusion Several forms of skin cancer are successfully treated with the topical drug Imiquimod, which activates pDCs through toll-like receptor 7 engagement. Additionally, pDC-based anticancer vaccines have shown encouraging results for the treatment of melanoma in early trials. Future studies regarding the contributions of pDCs to malignancy will likely afford many opportunities for immunotherapy strategies.

Keywords Cancer, immunity, pDC, plasmacytoid dendritic cells, tolerance, tumour.

Eur J Clin Invest 2015; 45 (S1): 1–8

Introduction

Plasmacytoid dendritic cells (pDCs) are a unique population of bone-marrow-derived immune cells that bridge the innate and adaptive immune systems. They are remarkable in that they are the only immune cell to serve two professional roles, one as interferon (IFN)-producing cells and the other as antigen-presenting cells (APCs). Although accounting for only 0·3–0·5% of peripheral blood cells, pDCs are responsible for over 95% of type I IFN produced by circulating lymphocytes [1]. Activation of pDCs and the subsequent production of IFN occur as the result of a signalling cascade that initiates through the receptor-ligand interactions of pattern recognition receptors (reviewed by Lombardi and Khaiboullina [2]). pDCs are primarily activated through the engagement of endosomally located toll-like receptors (TLR)-7 and TLR-9, by ssRNA [3,4] or nonmethylated and CpG DNA [5,6], respectively (Fig. 1a), which are common to microbial genomes, such as viruses or their replicative intermediates. pDCs are also known to produce type I IFN in response to double-stranded dsRNA, probably through the engagement of protein kinase R (PRK) [7], although their response to dsRNA is less well characterized. Similar to other TLRs, (with the notable exception of TLR-3), TLR-7 and TLR-9 utilize the universal adapter protein MyD88 (myeloid differentiation primary response 88), which acts via the constitutively expressed transcription factor IRF7 and the inflammatory transcription factor NF-κB, thereby initiating transcription of type I and III IFN, or inflammatory cytokines and chemokines, respectively [8–10]. Upon activation, pDCs also undergo phenotypic changes resulting in the upregulation of costimulatory molecules, including CD40, CD80, CD86. They ultimately develop into more 'conventional' dendritic cells (cDC) with classical DC morphology and the ability to present and cross-present antigens in the context of MHC and costimulatory molecules to naïve and memory T cells [11].

Over the last decade, our understanding of pDCs biology has greatly expanded but this expansion has also resulted in many unanswered questions. Indeed, it is now evident that pDCs play a much larger role in immunology than originally realized. In addition to their ability to produce IFN, pDCs contribute to

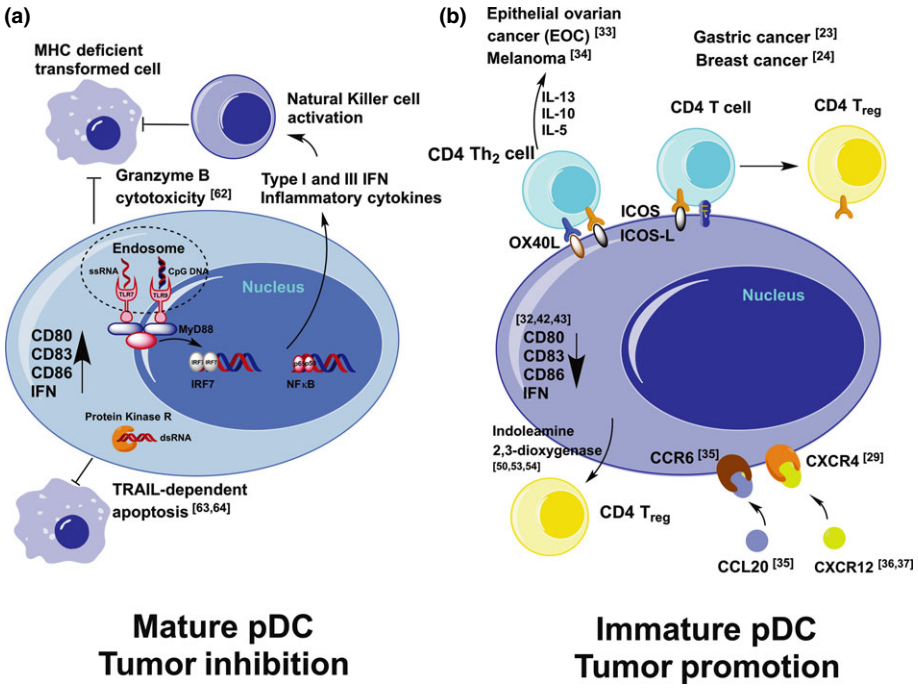

Figure 1 Plasmacytoid dendritic cell (pDC) involvement in tumour inhibition and promotion. (a) Activation of pDCs through the engagement of endosomally located toll-like receptors (TLR) 7 and 9 by ssRNA or nonmethylated CpG DNA, respectively, leads to the MyD88-dependent upregulation of type I interferon (IFN) and inflammatory cytokines as well as expression of costimulatory molecules such as CD80, CD83 and CD86. Type I IFN expression also occurs through the engagement of protein kinase R (PKR) by dsRNA. pDCs possess direct tumoricidal activity in a granzyme B and TRAIL-dependent manner and indirectly through the activation of natural killer (NK) cells by type I IFN. (b) Mechanisms suggested to explain tumour-associated pDC dysfunction include the recruitment of immature pDCs as characterized by the lack of expression of costimulatory molecules and tumour secretion of immunosuppressive factors. In addition to being immature, these pDCs are shown to promote tolerance by activating Tregs, express anti-inflammatory cytokines such as IL-13 and are refractory in IFN production.

tolerance but when dysregulated can also contribute to autoimmunity. Current research suggests that pDCs have the capacity to induce apoptosis of neoplastic cells and, therefore, may also contribute to cancer surveillance. Conversely, it has also been shown that the tolerogenic functions of pDCs may be utilized by tumours to their advantage, allowing them a way to evade the immune system. For these reasons, an understanding of pDC function in the context of neoplastic pathology and the tumour microenvironment will likely provide a greater understanding of malignancy in general and suggest potential treatment strategies.

Neoplastic progression and the tumour microenvironment

The historical paradigm of cancer development and propagation is based upon the presence of mutations that lead to cell cycle dysregulation. According to this model, a single mutation in a cell cycle gene allows the cell to grow uncontrolled whereby it rapidly expands to form a tumour. However, this model is an oversimplification in that as the tumour expands, it forms its own microenvironment that differs from that of healthy non-malignant tissue [12]. Indeed, cross-talk between stromal and epithelial cells is essential for maintaining homoeostasis of malignant as well as non-malignant tissue [13,14]. In the last three decades, our knowledge regarding the role of immune effector cells in maintaining a protumorigenic microenvironment has increased substantially. For instance, tumour-derived colony-stimulating factor-1 (CSF-1), VEGF and endothelial monocyte activating polypeptide II (EMARII) have been shown to facilitate the infiltration of tumour tissue by monocytes [15–17]. Within the tumour, monocyte-derived macrophages polarize into the M2 stage, which is strongly associated with proangiogenic and protumorigenic properties [18,19]. Furthermore, tumour-associated macrophages contribute to an immunosuppressive environment by releasing interleukin (IL)-10 and TGF-β [20] and additionally, promote the infiltration of T regulatory cells (Tregs) by releasing the

chemoattractant CCL22 [21,22]. A positive correlation has been observed between inducible costimulator (ICOS)-expressing Tregs and pDCs in the peripheral blood and peritumour tissue of subjects with gastric cancer [23] (Fig. 1b). Additionally, ICOS-driven interaction between CD4+ T cells and pDCs has been reported to lead to the upregulation of Tregs and IL-10 secretion in breast tumours [24]. These observations underscore the contributions of immune effector cells to an immunosuppressive tumour microenvironment, thus supporting the maintenance and propagation of malignancy.

Plasmacytoid dendritic cells, immunity and cancer

All DCs are professional APCs with the capacity to prime and activate naïve T lymphocytes [25]. By controlling the outcome of antigen presentation to T cells, DCs also play a central role in the maintenance of peripheral tolerance. Through the activation of pattern recognition receptors, such as TLRs, they also produce cytokines such as interferons and interleukins, thus modulating the balance between humoral immunity, cell-mediated immunity and tolerance [26]. For these reasons, it is not surprising that DCs may play a pivotal role in antittumour immunity. Involvement of pDCs in neoplastic disorders became evident upon the observation that several tumours including ovarian, head and neck, and breast tumours and primary melanoma are infiltrated with pDCs [27–32]. In some instances, the presence of infiltrating pDCs is associated with a poor prognosis; for example, while investigating epithelial ovarian cancer (EOC), Conrad and co-workers observed that a significant number of Foxp3+ Tregs present in the tumour microenvironment expressed ICOS [33]. They further observed that the ability of these cells to suppress T-cell proliferation was strictly dependent on ICOS-L costimulation provided by infiltrating pDCs and therefore suggested that pDCs and ICOS+ Foxp3+ Tregs were strong predictors of EOC progression. As a further example, Aspord *et al.* reported that Th2-promoting pDCs were associated with the progression of melanoma and that the frequency of IL-5, 10 and 13-producing T cells in melanoma cases was correlated with a high proportions of OX40L- and ICOSL-expressing pDCs [34].

Dendritic cells play a central role in orchestrating immune responses, and numerous studies have reported that tumour tissue is often infiltrated with various populations of DCs including pDCs. For example, as previously stated, pDCs have been reported to be among the cellular infiltrate of several tumours [27–32]. It is believed that the recruitment of pDCs into tumour tissue is governed by chemokines secreted by neoplastic cells. Zou *et al.*, as well as others, have reported that tumours infiltrated with pDCs express high levels of chemokines such as CXCL12 (stromal cell-derived factor 1) and CCL20 (macrophage inflammatory protein-3) [29,32,35]. Zou and co-workers additionally reported that tumour-derived pDCs express high levels of CXCR4 [29], the specific receptor for CXCL12 [36,37]. Charles *et al.* reported that tumour-associated pDCs express high levels of the chemokine receptor CCR6, the receptor for CCL20 [35], a requirement for the rapid recruitment of dendritic cells into tissue [38]. Indeed, the multiple receptor-ligand interactions that occur between tumour cells and immune effector cells contribute to the complex microenvironment that allows tumours to maintain their own persistence.

pDCs are essential for recognition of altered self-antigens and for triggering immune responses directed towards transformed cell. Therefore, it would be expected that the increased presence of pDCs in tumour tissue should promote immune recognition of tumour antigens and, in turn, lead to tumour rejection. Contrary to this supposition and unexpectedly, increased pDC tumour infiltration is often associated with tumour progression and persistence [24,39]. Furthermore, it has been shown that increased pDC infiltration is associated with poor prognosis in some cancer cases [40,41]. Therefore, it has been suggested that tumour-associated pDCs are often incompetent with respect to tumour-specific immune surveillance.

Several mechanisms have been suggested to explain tumour-associated pDC dysfunction including the recruitment of immature pDCs, promotion of pDC tolerance and tumour secretion of immunosuppressive factors. Numerous studies have shown that tumour-associated pDCs are immature as characterized by the lack of expression of costimulatory molecules such as CD80, CD83 and CD86 [32,42,43]. In addition to being immature, these pDCs are shown to be defective in IFNα production [32,44] and it has been suggested that defective IFNα production is the result of a downregulation of Flt3, TLR9 or IRF7 [44–47]. Tsukamoto *et al.* [48] proposed that tumour-associated immunoglobulin-like transcript 7 ligands (ILT7L) can downregulate IFNα production in pDCs via interaction with the ILT7 receptor. IFNα is a pleotropic cytokine with strong tumour inhibitory activity [49]. Therefore, by producing less IFNα, pDCs may significantly impair local immune surveillance allowing tumours to escape IFNα-associated immune responses. Several studies have suggested that tumour-associated pDCs are indeed tolerogenic [28,50]. For example, animal tumour models have shown that tumour-infiltrating pDCs can activate mature Tregs [51,52]. Additionally, it is well documented that malignant cells and tumour-associated pDCs release indoleamine 2,3-dioxygenase (IDO) which is a powerful promoter of Treg activation, and can lead to anergy, thus allowing tumour cell to escape immune surveillance [50,53,54].

Although pDC infiltration of tumours is often associated with disease progression, their activation with TLR-agonists is proving to be an effective treatment for some forms of neoplasm. For instance, the topical treatment of basal cell carcinoma, superficial squamous cell carcinoma and some superficial malignant melanomas, with the synthetic TLR-7 agonist Imiquimod, has been shown to lead to the increased infiltration of activated pDCs and a significant reduction in neoplastic cells and in some cases, a complete regression [31,55–58].

Antineoplastic functions of pDCs

DCs have the potential to invoke antitumour immunity in multiple ways. Similar to cytotoxic CD8 T lymphocytes (CTLs), natural killer (NK) cells and gamma/delta T cells; DCs have the capacity for direct cytotoxic killing of susceptible target cells such as virus-infected cells and transformed cells. The focus of our discussion is the contribution of pDCs to anticancer immunity; notwithstanding, significant body of research also addresses the cytotoxic capacity of cDCs. These topics are excellently reviewed by Tel *et al.*, and Larmonier *et al.*, with respect to humans and animal models [59,60] and thus we will only discuss their tumoricidal activity in conjunction with, or in comparison to pDCs.

Classic cytotoxic cells, such as NK cells and CTLs, express perforin (PRF1) and the proapototic enzyme granzyme B (GZMB). Although initially believed that PRF1 was required for entry of GZMB into target cells, current research suggests that both proteins may enter cells through an alternative mechanism. For instance, Veugelers and colleagues proposed a mannose 6-phosphate receptor as a potential entry mechanism for PRF1 and GRZB [61]. The definitive role for pDC-GRZB is currently the subject of ongoing investigations. However, as pDCs do not express PRF1, an alternative PRF1-independent entry method would support the possibility of pDC cytotoxicity in a GZMB-dependent but PRF1-independent manner. Indeed, using a human asthma model of segmental allergen challenge, Bratke and co-workers reported that pDCs upregulate GRZB in response to IL-3 and additionally showed that IL-3 activated pDCs killed MHC deficient K562 cells [62]. Furthermore, they reported that the observed killing was abrogated in the presence of GRZB and caspase inhibitors. Interestingly, they also observed that engagement of the TLR-7 or -9 receptor suppressed GRZB expression, suggesting that the classical IFN-induced pathway of pDCs is not involved in GRZB-associated cytotoxicity. Tel *et al.* reported that human pDCs activated with the preventative vaccine to tick-borne encephalitis virus FSME upregulated the neural cell adhesion marker CD56, a classic NK marker, and were empowered with the tumoricidal ability to lyse K562 and Daudi cells in a contact-dependent manner [63]. They additionally reported that the expression of CD56 on the surface of pDCs coincided with elevated expression of programmed death-ligand 1 (PD-L1), GRZB and TNF-related apoptosis-inducing ligand (TRAIL).

TRAIL-dependent apoptosis has been implicated in the tumoricidal capacity of pDCs by other researchers as well. For instance, Stary and co-workers reported that, upon treatment of basal cell carcinoma with Imiquimod, a cellular infiltrate of GRZB and PRF1 positive cDCs and TRAIL positive pDCs was observed [64]. However, in contrast to the observations of Bratke *et al.*, the contribution of pDC killing was strictly TRAIL dependent, as TRAIL neutralizing antibody abrogated the killing of TRAIL-sensitive Jurket cells. Consistent with the observations of Stary *et al.*, Kalb and co-workers reported that pDCs stimulated with agonists for TLR-7 and 9, but not other TLRs, upregulated the surface expression of TRAIL in a type I IFN-dependent manner [65]. They additionally reported that pDCs treated with TLR7/9 agonists as well as pDCs treated with type I IFN efficiently lysed Jurkat cells, as well as the melanoma cell lines SKMel2 and WM793, in a TRAIL and contact-dependent manner. Using a mouse model of melanoma, Drobits and co-workers showed that topical Imiquimod treatment resulted in tumour clearance in a TLR7/MyD88-dependent and IFN-α/β receptor 1-dependent manner, with a concomitant upregulation of the chemokine CCL2 in mast cells [66]. They additionally observed that Imiquimod treatment promoted the secretion of both TRAIL and GRZB and that blocking these molecules led to impaired pDC-mediated tumour killing. These data strongly implicate both TRAIL and GRZB in pDC-mediated tumour killing and further suggest that the tumoricidal ability of pDCs is independent of adaptive immunity.

pDCs as potential targets in cancer immunotherapy

CTLs are considered to be the most critical mediators of anticancer immune responses, and CTL infiltration of tumours is typically associated with a positive diagnostic outcome [67,68]. The use of immunomodulating drugs to increase CTL responses has been shown to be an effective strategy for improving the induction of long-term memory CTLs. For instance, one strategy targets the blockade of the inhibitory receptors such as the cytotoxic T lymphocyte-associated antigen 4, the programmed death-1 receptor (PD-1) or its ligand, PD-L1. This approach is often referred to as 'immune-check-point blockade'. The use of anti-PD1 antibodies in combination with the anti-B cell drug rituximab has led to encouraging results both in preclinical models and in clinical applications [69,70]. However, the nonspecific nature of these 'check point inhibiting' drugs and the broad mechanism by which they exert

their actions can lead to activation of autoreactive T cells and, in turn, lead to potentially severe side effects [71,72]. DC therapies are an attractive alternative to check point-inhibiting drugs in that they have few side effects, and natural DC therapy is generally less costly.

Ex vivo DCs are capable of inducing CTL responses against tumours when loaded with tumour-associated antigens and given as a vaccine. Therefore, the primary goal of cancer vaccine immunotherapy is the induction of long-term memory CTLs that are capable of facilitating immune surveillance and promoting tumour rejection. Although the use of cancer vaccines to generate antitumour immune responses is theoretically promising and appears fairly straightforward, the clinical success of such vaccines has been less than encouraging [73]. Although previous studies have largely employed monocyte-derived DCs (moDCs) for this purpose, a pioneering study conducted in the laboratory of Dr. Jolanda De Vries utilized activated pDCs preloaded with tumour-associated antigens to vaccinate subjects with melanoma [74]. Although the overall magnitude of antimelanoma immune responses was comparable to that of previous moDC trials, a number of encouraging observations were made as a result of this study. The pDC vaccine produced a systemic type I IFN response, which is critical to NK activation and subsequent inhibition of tumour metastasis [75]. Additionally, pDCs were observed to migrate efficiently to the lymph nodes and, subsequently, T-cell clones with high avidity could be identified after vaccination, indicative of a strong functional response. Lastly and most importantly, the overall survival of subjects treated with the vaccine greatly increased when compared to matched controls that only received a standard chemotherapy treatment. With regard to the mechanism of the observed efficacy, one could speculate that the improved treatment outcome may have been the result of pDC-mediated activation of innate immune cells such as NK cells, or perhaps T cells induced by pDCs may be more potent immune effectors. Nevertheless, these observations clearly suggest that pDC-based anticancer vaccines will likely provide advantages over moDC vaccinations or may even supplement moDC vaccinations when used in combination therapy.

Concluding remarks

The current model of tumour neogenesis holds that the tumour microenvironment provides favourable conditions that support malignant cell growth and propagation, while at the same time, allows them to evade the immune system. pDCs that infiltrate tumours are often dysfunctional and, accordingly, do not produce IFNα. Furthermore, they often display an immature or naïve phenotype and promote a tolerogenic microenvironment through the activation of Tregs. In this context, pDCs likely contribute to neoplastic homoeostasis and, accordingly, represent a very attractive target in cancer immunotherapy. Indeed, activating pDCs with the TLR-7 agonist, Imiquimod is highly effective in treating some forms of skin cancer and exemplifies the potential impact of pDC immunity in neoplastic disease. Additionally, pDCs have been shown to have tumoricidal properties in culture; therefore, potentiating this ability in vivo may prove to be an effective treatment strategy. Future studies regarding the contributions of pDCs to malignancy will likely afford many opportunities for immunotherapy strategies.

Acknowledgements

We are indebted to Dr. Doug Redelman for his careful review of this manuscript. Drs. Lombardi and Khaiboullina are supported in part by grants from the National Institutes of Health (AI078234), the Department of Defense (GW100091), and the Program of Competitive Growth of Kazan Federal University supports work conducted in the laboratory of Dr. Rizvanov.

Conflict of interest

The authors declare no conflict of interests.

Address

Department of Biochemistry and Molecular Biology, University of Nevada School of Medicine, WPI, 1664 N Virginia Street MS 0552, Reno, NV, 89557, USA (V. C. Lombardi, S. F. Khaiboullina); Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, Kremlevskaya St 18, Kazan, 420008, Tatarstan, Russian Federation (V. C. Lombardi, S. F. Khaiboullina, A. A. Rizvanov).

Correspondence to: Vincent C. Lombardi, Ph.D., Department of Biochemistry and Molecular Biology, University of Nevada School of Medicine, WPI, 1664 N Virginia Street MS 0552, Reno, NV, 89557, USA. Tel.: +1 7756828271; fax: 7756828258; e-mail: vlombardi@medicine.nevada.edu and
Albert A. Rizvanov, Ph.D., D.Sc., leading scientist, Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, Kremlevskaya St 18, Kazan, 420008, Tatarstan, Russian Federation. Tel.: +7 843 2337843; fax: +7 843 2337814; e-mail: Albert.Rizvanov@kpfu.ru

References

1 Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S *et al*. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 1999;**284**: 1835–7.
2 Lombardi VC, Khaiboullina SF. Plasmacytoid dendritic cells of the gut: relevance to immunity and pathology. *Clin Immunol* 2014;**153**:165–77.

3 Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004;**303**:1529–31.
4 Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S *et al.* Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004;**303**:1526–9.
5 Kadowaki N, Antonenko S, Liu YJ. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c- type 2 dendritic cell precursors and CD11c+ dendritic cells to produce type I IFN. *J Immunol* 2001;**166**:2291–5.
6 Kerkmann M, Rothenfusser S, Hornung V, Towarowski A, Wagner M, Sarris A *et al.* Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. *J Immunol* 2003;**170**:4465–74.
7 Ruscanu S, Pascale F, Bourge M, Hemati B, Elhmouzi-Younes J, Urien C *et al.* The double-stranded RNA bluetongue virus induces type I interferon in plasmacytoid dendritic cells via a MYD88-dependent TLR7/8-independent signaling pathway. *J Virol* 2012;**86**:5817–28.
8 Hochrein H, O'Keeffe M, Wagner H. Human and mouse plasmacytoid dendritic cells. *Hum Immunol* 2002;**63**:1103–10.
9 Krug A, Rothenfusser S, Hornung V, Jahrsdorfer B, Blackwell S, Ballas ZK *et al.* Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol* 2001;**31**:2154–63.
10 Proietto AI, O'Keeffe M, Gartlan K, Wright MD, Shortman K, Wu L *et al.* Differential production of inflammatory chemokines by murine dendritic cell subsets. *Immunobiology* 2004;**209**:163–72.
11 Hellman P, Eriksson H. Early activation markers of human peripheral dendritic cells. *Hum Immunol* 2007;**68**:324–33.
12 Bizzarri M, Cucina A. Tumor and the microenvironment: a chance to reframe the paradigm of carcinogenesis? *Biomed Res Int* 2014;**2014**:934038.
13 Vosseler S, Mirancea N, Bohlen P, Mueller MM, Fusenig NE. Angiogenesis inhibition by vascular endothelial growth factor receptor-2 blockade reduces stromal matrix metalloproteinase expression, normalizes stromal tissue, and reverts epithelial tumor phenotype in surface heterotransplants. *Cancer Res* 2005;**65**:1294–305.
14 Barcellos-Hoff MH, Ravani SA. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer Res* 2000;**60**:1254–60.
15 Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* 2001;**193**:727–40.
16 Kao J, Houck K, Fan Y, Haehnel I, Libutti SK, Kayton ML *et al.* Characterization of a novel tumor-derived cytokine. Endothelial-monocyte activating polypeptide II. *J Biol Chem* 1994;**269**:25106–19.
17 Balkwill F. Cancer and the chemokine network. *Nat Rev Cancer* 2004;**4**:540–50.
18 Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* 2010;**141**:39–51.
19 Zaynagetdinov R, Sherrill TP, Polosukhin VV, Han W, Ausborn JA, McLoed AG *et al.* A critical role for macrophages in promotion of urethane-induced lung carcinogenesis. *J Immunol* 2011;**187**:5703–11.
20 Kryczek I, Zou L, Rodriguez P, Zhu G, Wei S, Mottram P *et al.* B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *J Exp Med* 2006;**203**:871–81.
21 Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;**10**:942–9.
22 Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H *et al.* CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. *Int J Cancer* 2008;**122**:2286–93.
23 Huang XM, Liu XS, Lin XK, Yu H, Sun JY, Liu XK *et al.* Role of plasmacytoid dendritic cells and inducible costimulator-positive regulatory T cells in the immunosuppression microenvironment of gastric cancer. *Cancer Sci* 2014;**105**:150–8.
24 Faget J, Bendriss-Vermare N, Gobert M, Durand I, Olive D, Biota C *et al.* ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive CD4+ T cells. *Cancer Res* 2012;**72**:6130–41.
25 Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 2007;**7**:19–30.
26 Villadangos JA, Schnorrer P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat Rev Immunol* 2007;**7**:543–55.
27 Salio M, Cella M, Vermi W, Facchetti F, Palmowski MJ, Smith CL *et al.* Plasmacytoid dendritic cells prime IFN-gamma-secreting melanoma-specific CD8 lymphocytes and are found in primary melanoma lesions. *Eur J Immunol* 2003;**33**:1052–62.
28 Hartmann E, Wollenberg B, Rothenfusser S, Wagner M, Wellisch D, Mack B *et al.* Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res* 2003;**63**:6478–87.
29 Zou W, Machelon V, Coulomb-L'Hermin A, Borvak J, Nome F, Isaeva T *et al.* Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat Med* 2001;**7**:1339–46.
30 Treilleux I, Blay JY, Bendriss-Vermare N, Ray-Coquard I, Bachelot T, Guastalla JP *et al.* Dendritic cell infiltration and prognosis of early stage breast cancer. *Clin Cancer Res* 2004;**10**:7466–74.
31 Palamara F, Meindl S, Holcmann M, Luhrs P, Stingl G, Sibilia M. Identification and characterization of pDC-like cells in normal mouse skin and melanomas treated with imiquimod. *J Immunol* 2004;**173**:3051–61.
32 Vermi W, Bonecchi R, Facchetti F, Bianchi D, Sozzani S, Festa S *et al.* Recruitment of immature plasmacytoid dendritic cells (plasmacytoid monocytes) and myeloid dendritic cells in primary cutaneous melanomas. *J Pathol* 2003;**200**:255–68.
33 Conrad C, Gregorio J, Wang YH, Ito T, Meller S, Hanabuchi S *et al.* Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. *Cancer Res* 2012;**72**:5240–9.
34 Aspord C, Leccia MT, Charles J, Plumas J. Plasmacytoid dendritic cells support melanoma progression by promoting Th2 and regulatory immunity through OX40L and ICOSL. *Cancer Immunol Res* 2013;**1**:402–15.
35 Charles J, Di Domizio J, Salameire D, Bendriss-Vermare N, Aspord C, Muhammad R *et al.* Characterization of circulating dendritic cells in melanoma: role of CCR6 in plasmacytoid dendritic cell recruitment to the tumor. *J Invest Dermatol* 2010;**130**:1646–56.
36 Rollins BJ. Chemokines. *Blood* 1997;**90**:909–28.
37 Tilton B, Ho L, Oberlin E, Loetscher P, Baleux F, Clark-Lewis I *et al.* Signal transduction by CXC chemokine receptor 4. Stromal cell-derived factor 1 stimulates prolonged protein kinase B and

extracellular signal-regulated kinase 2 activation in T lymphocytes. *J Exp Med* 2000;**192**:313–24.
38 Le Borgne M, Etchart N, Goubier A, Lira SA, Sirard JC, van Rooijen N, *et al.* Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8+ T cell crosspriming in vivo. *Immunity* 2006;**24**:191–201.
39 Le Mercier I, Poujol D, Sanlaville A, Sisirak V, Gobert M, Durand I *et al.* Tumor promotion by intratumoral plasmacytoid dendritic cells is reversed by TLR7 ligand treatment. *Cancer Res* 2013;**73**:4629–40.
40 Labidi-Galy SI, Treilleux I, Goddard-Leon S, Combes JD, Blay JY, Ray-Coquard I *et al.* Plasmacytoid dendritic cells infiltrating ovarian cancer are associated with poor prognosis. *Oncoimmunology* 2012;**1**:380–2.
41 Faget J, Sisirak V, Blay JY, Caux C, Bendriss-Vermare N, Menetrier-Caux C. ICOS is associated with poor prognosis in breast cancer as it promotes the amplification of immunosuppressive CD4 T cells by plasmacytoid dendritic cells. *Oncoimmunology* 2013;**2**: e23185.
42 Perrot I, Blanchard D, Freymond N, Isaac S, Guibert B, Pacheco Y *et al.* Dendritic cells infiltrating human non-small cell lung cancer are blocked at immature stage. *J Immunol* 2007;**178**:2763–9.
43 Beckebaum S, Zhang X, Chen X, Yu Z, Frilling A, Dworacki G *et al.* Increased levels of interleukin-10 in serum from patients with hepatocellular carcinoma correlate with profound numerical deficiencies and immature phenotype of circulating dendritic cell subsets. *Clin Cancer Res* 2004;**10**:7260–9.
44 Saulep-Easton D, Vincent FB, Le Page M, Wei A, Ting SB, Croce CM *et al.* Cytokine-driven loss of plasmacytoid dendritic cell function in chronic lymphocytic leukemia. *Leukemia* 2014;**28**:2005–15.
45 Jensen TO, Schmidt H, Moller HJ, Donskov F, Hoyer M, Sjoegren P *et al.* Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer* 2012;**118**:2476–85.
46 O'Donnell RK, Mick R, Feldman M, Hino S, Wang Y, Brose MS *et al.* Distribution of dendritic cell subtypes in primary oral squamous cell carcinoma is inconsistent with a functional response. *Cancer Lett* 2007;**255**:145–52.
47 Sisirak V, Vey N, Goutagny N, Renaudineau S, Malfroy M, Thys S *et al.* Breast cancer-derived transforming growth factor-beta and tumor necrosis factor-alpha compromise interferon-alpha production by tumor-associated plasmacytoid dendritic cells. *Int J Cancer* 2013;**133**:771–8.
48 Tsukamoto N, Okada S, Onami Y, Sasaki Y, Umezawa K, Kawakami Y. Impairment of plasmacytoid dendritic cells for IFN production by the ligand for immunoglobulin-like transcript 7 expressed on human cancer cells. *Clin Cancer Res* 2009;**15**:5733–43.
49 McCarty MF, Bielenberg D, Donawho C, Bucana CD, Fidler IJ. Evidence for the causal role of endogenous interferon-alpha/beta in the regulation of angiogenesis, tumorigenicity, and metastasis of cutaneous neoplasms. *Clin Exp Metastasis* 2002;**19**:609–15.
50 Watkins SK, Zhu Z, Riboldi E, Shafer-Weaver KA, Stagliano KE, Sklavos MM *et al.* FOXO3 programs tumor-associated DCs to become tolerogenic in human and murine prostate cancer. *J Clin Invest* 2011;**121**:1361–72.
51 Sharma MD, Baban B, Chandler P, Hou DY, Singh N, Yagita H *et al.* Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. *J Clin Invest* 2007;**117**:2570–82.
52 Ito T, Yang M, Wang YH, Lande R, Gregorio J, Perng OA *et al.* Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med* 2007;**204**:105–15.
53 Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. *J Clin Invest* 2007;**117**:1147–54.
54 Munn DH, Sharma MD, Hou D, Baban B, Lee JR, Antonia SJ *et al.* Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J Clin Invest* 2004;**114**:280–90.
55 Preynat-Seauve O, Schuler P, Contassot E, Beermann F, Huard B, French LE. Tumor-infiltrating dendritic cells are potent antigen-presenting cells able to activate T cells and mediate tumor rejection. *J Immunol* 2006;**176**:61–7.
56 Beutner KR, Geisse JK, Helman D, Fox TL, Ginkel A, Owens ML. Therapeutic response of basal cell carcinoma to the immune response modifier imiquimod 5% cream. *J Am Acad Dermatol* 1999;**41**:1002–7.
57 Urosevic M, Dummer R, Conrad C, Beyeler M, Laine E, Burg G *et al.* Disease-independent skin recruitment and activation of plasmacytoid predendritic cells following imiquimod treatment. *J Natl Cancer Inst* 2005;**97**:1143–53.
58 van Egmond S, Hoedemaker C, Sinclair R. Successful treatment of perianal Bowen's disease with imiquimod. *Int J Dermatol* 2007;**46**:318–9.
59 Tel J, Anguille S, Waterborg CE, Smits EL, Figdor CG, de Vries IJ. Tumoricidal activity of human dendritic cells. *Trends Immunol* 2014;**35**:38–46.
60 Larmonier N, Fraszczak J, Lakomy D, Bonnotte B, Katsanis E. Killer dendritic cells and their potential for cancer immunotherapy. *Cancer Immunol Immunother* 2010;**59**:1–11.
61 Veugelers K, Motyka B, Goping IS, Shostak I, Sawchuk T, Bleackley RC. Granule-mediated killing by granzyme B and perforin requires a mannose 6-phosphate receptor and is augmented by cell surface heparan sulfate. *Mol Biol Cell* 2006;**17**:623–33.
62 Bratke K, Nielsen J, Manig F, Klein C, Kuepper M, Geyer S *et al.* Functional expression of granzyme B in human plasmacytoid dendritic cells: a role in allergic inflammation. *Clin Exp Allergy* 2010;**40**:1015–24.
63 Tel J, Smits EL, Anguille S, Joshi RN, Figdor CG, de Vries IJ. Human plasmacytoid dendritic cells are equipped with antigen-presenting and tumoricidal capacities. *Blood* 2012;**120**:3936–44.
64 Stary G, Bangert C, Tauber M, Strohal R, Kopp T, Stingl G. Tumoricidal activity of TLR7/8-activated inflammatory dendritic cells. *J Exp Med* 2007;**204**:1441–51.
65 Kalb ML, Glaser A, Stary G, Koszik F, Stingl G. TRAIL(+) human plasmacytoid dendritic cells kill tumor cells in vitro: mechanisms of imiquimod- and IFN-alpha-mediated antitumor reactivity. *J Immunol* 2012;**188**:1583–91.
66 Drobits B, Holcmann M, Amberg N, Swiecki M, Grundtner R, Hammer M *et al.* Imiquimod clears tumors in mice independent of adaptive immunity by converting pDCs into tumor-killing effector cells. *J Clin Invest* 2012;**122**:575–85.
67 Anichini A, Fossati G, Parmiani G. Clonal analysis of cytotoxic T-lymphocyte response to autologous human metastatic melanoma. *Int J Cancer* 1985;**35**:683–9.
68 Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;**313**:1960–4.
69 Berger R, Rotem-Yehudar R, Slama G, Landes S, Kneller A, Leiba M *et al.* Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. *Clin Cancer Res* 2008;**14**:3044–51.

70 Armand P, Nagler A, Weller EA, Devine SM, Avigan DE, Chen YB *et al.* Disabling immune tolerance by programmed death-1 blockade with pidilizumab after autologous hematopoietic stem-cell transplantation for diffuse large B-cell lymphoma: results of an international phase II trial. *J Clin Oncol* 2013;**31**:4199–206.
71 Dennis LK. Analysis of the melanoma epidemic, both apparent and real: data from the 1973 through 1994 surveillance, epidemiology, and end results program registry. *Arch Dermatol* 1999;**135**:275–80.
72 Robert C, Thomas L, Bondarenko I, O'Day S, M DJ, Garbe C *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011;**364**:2517–26.
73 Kono K. Current status of cancer immunotherapy. *J Stem Cells Regen Med* 2014;**10**:8–13.
74 Tel J, Aarntzen EH, Baba T, Schreibelt G, Schulte BM, Benitez-Ribas D *et al.* Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. *Cancer Res* 2013;**73**:1063–75.
75 Kim A, Noh YW, Kim KD, Jang YS, Choe YK, Lim JS. Activated natural killer cell-mediated immunity is required for the inhibition of tumor metastasis by dendritic cell vaccination. *Exp Mol Med* 2004;**36**:428–43.

